

Can Soviet science be rescued?

Soviet science is on the verge of collapse. That is the message from Moscow in the past few days. The gloomy news is plausible. The rest of the world must do what it can to help, but with discrimination.

ONE thing is sure about science now in what, for want of a better name, is still called the Soviet Union. Science is quickly coming to an end. In the past few days, several appeals for help have been circulating in the West, while the Moscow State University has made a public appeal for rescue. And Soviet visitors to the West now spend their time knocking on the doors of charitable foundations, describing the causes of the impending collapse and pleading, again, for help. The case is at once chilling and heartrending. That the rest of the world must help cannot be denied. The questions to be answered are simply how, on what scale and how quickly.

Little imagination is needed to tell why imminent collapse is likely. The circumstances of personal life in the Soviet Union are a sufficient explanation. People may enjoy a freedom unimagined in Stalin's time, but freedom will not buy bread. The salaries of those who perform essential services have necessarily increased as the state's powers of job direction have withered away, but run-of-the-mill researchers, whose contribution to the here and now is less immediate, are still paid the 300 to 600 rubles a month they earned in Stalin's time — an average of, say, \$10 a month at the now-official exchange rate, half as much at the street rate. Bus drivers earn twice as much or more. With the price of everything on the rise and with black markets everywhere replacing those of the official kind, most people must put personal and family survival ahead of research. They have no choice.

Over last weekend, the outlook seemed even more bleak. On Thursday, 28 November, the Soviet central bank announced that it had run out of funds, and would not be able to pay this month's salaries to employees of the centre (which most researchers still are) unless the Soviet Parliament were to relax its refusal to let the bank print a further 90,000 million rubles. But now Mr Boris Yeltsin's Russian government has stepped in, promising to meet the central government's payroll. That will avoid some hardship in the month ahead, but at the price of giving Yeltsin even more control over the centre. The future of Soviet science will be shaped by his government's undeclared opinion of it.

That will not soon be clarified. Turmoil bordering on chaos has pushed research onto every back-burner. The bureaucracy, or such of it as remains, has more urgent things on its mind than making sure that there are resources to keep research laboratories productive. Eco-

nomie reform is rightly the most urgent need. Unless it succeeds, and quickly (which, dangerously, means some years), chaos will give way to anarchy and there will be no science anyway. Faced with such a prospect, governments are right to concentrate only on what matters most. In such circumstances, to ask that Yeltsin should put the rescue of Soviet science high on his list is to ask too much.

The rest of the world should understand that there is no historical precedent for what is happening in the Soviet Union. The collapse of the Roman Empire was, by comparison, a leisurely business, occupying centuries. The Austro-Hungarian Empire came to an abrupt end (in 1914), but only after a long period during which its central authority had steadily been eroded. The Soviet Union, by contrast, has been transformed in six years from one of the most conservative of systems, renowned both for financial probity and civil order (repressively secured) into one in which the most primitive of the 'means of exchange' — barter — has become an essential supplement to the use of currency, in which the central bank has woken up to find its once-fabulous stocks of gold exhausted and in which the centrifugal tendencies of ethnic (or, more often, linguistic) nationalism have made the most influential institutions of the stalinist state pawns in the republics' scramble for influence among each other.

In the circumstances, it must be allowed that the Soviet Academy of Sciences has done better than expected in the past six years at insulating some of its laboratories from the chaos in which they are embedded. But the academy and its dependent laboratories cannot be stronger than Mikhail Gorbachev and his government, whose future influence hangs on a still nonexistent union treaty. So now the academy (see page 342) is itself in limbo, in transition to a new status under Yeltsin's wing as the Russian Academy of Sciences. (Quite what that means may become clearer at a meeting to be held next week in Moscow.) Yet even Yeltsin is not made of money, while economic reform is the most urgent need. Things must get even worse if they are ever to get better.

What, in these circumstances, can researchers do? Most rational responses involve some version of getting out. The hunt for overseas posts is now ceaseless. The trickle of those leaving the Soviet Union would be a flood if only there were more posts in the West and elsewhere. In the Soviet Union as in other places, the more able are those most able to quit. Although the numbers are still



only hundreds, they are enough substantially to damage the future health of Soviet science. (That some of those who feel compelled to emigrate may be people with a background in defence research will mean trouble for others than the Soviet Union.) But the flight of talent overseas, serious though it is, is not the most serious threat.

Nor, in the Soviet Union, is the escape route 'into industry', much used elsewhere in the world, open in any ordinary sense. The Soviet Union's production plants, not yet privatized, are not accustomed to carrying out their own research and development, which has been the responsibility, in the stalinist tradition, of ministry-run research establishments. Neither the plant managers (now as short of funds as any public institutions) nor the research establishments (cramped by the bureaucratic tradition) are well-placed to make constructive deals with each other. Yet radical ideas are now more needed than ever. Some research laboratories, or groups within them, should at least consider whether to turn themselves into productive enterprises of some kind. There has also been talk, at some research institutes, of negotiating research contracts with industrial companies in the West. The attractions of hard-currency earnings are obvious, but the difficulties are immense, and success must be thinly spread and sporadic and cannot in the long run rescue Soviet science.

That is why the most likely end-point of the present squeeze on Soviet science is that people will be forced out of research and into other jobs that offer at least the chance of personal survival, bus-driving for example. It goes without saying, of course, that the Soviet system of research is bloated with uncreative people and larger, numerically and otherwise, than even a prosperous economy could support. Some slimming down is overdue. But as with emigration, the most able will be the most mobile. The natural rump of a research enterprise shrunk by economic forces alone will probably be moribund.

Would that be a calamity? Of course, both for the Soviet Union and the rest of us. The wellbeing of Soviet science will determine the character of the states emerging from the present upheaval. Times may now be so desperate that research can be neglected, perhaps for several years, but who will benefit if the end-result is a group of newly constituted countries unable, for lack of teachers, to renew its stock of skill and unable, for lack of skill, to run industrial enterprises in a modern way? The impoverishment of the Soviet Union is not a recipe for victory in some imagined prolongation of the cold war, but a recipe for disaster all round — further impoverishment of the people living there, unknown dangers for the rest of us.

A more particular argument bears on the present plight of Soviet science, which is integrally a part of international science. Mendelev is a pre-Stalin name to conjure with, but one of the remarkable features of the past 70 years is that Soviet research has contributed powerfully and consistently to the general understanding. Where would physics be without Landau, or cosmology without

Zeldovich (both now dead)? And the late Andrei Sakharov is only the best-known among those in the Soviet Union who have used their influence over several decades to bring about the *rapprochement* with the West that is cause of the present upheaval. The rest of science cannot let Soviet science now collapse without cutting off a part of itself, and it cannot turn its back on the colleagues with whom it has collaborated in the six Gorbachev years without great shame.

But how to help? On what scale? And when? The objective must be to keep as many able people as possible in active research and where they are. The needs are various. Some need small quantities of supplies and equipment. Others need the opportunity to travel briefly, perhaps to forge collaborations overseas. Professional journals (or the lack of them) are generally a headache, making Soviet researchers too often feel that they are working in the dark. And as things are, even the most able people will not be able to work creatively if they are not insulated from the hardships of ordinary life by grants of spendable money, however small by others' standards. The need will persist for three years: either economic reform will by then be working, or chaos will have given way to anarchy.

So why not transfer a lot of money to the Soviet Union? Because that would be a waste of money, and would serve no purpose. Saving Soviet science does not mean relieving every Soviet scientist from hardship, but identifying the able people and arranging that they alone win support. In the United States, the National Science Foundation has a special scheme for awarding grants to research projects involving collaboration between US and Soviet groups; this is an excellent recipe, but one that cannot cover the numbers of those at risk of being squeezed out of research. Moreover, there are few institutions in the Soviet Union — perhaps only a few outstanding institutes — that could be trusted to spend substantial sums of money only on the most able people. Principles of fairness, admirable in normal times, would get in the way, as would old-fashioned nepotism.

So there needs to be a means by which well-wishers outside the Soviet Union, of whom there are a great many, could be helped to identify the able young people on whom the future of the Soviet Union rests. In what would be a break with Soviet tradition, outsiders would have to play an important part in identifying the people (necessarily young) deserving of support. But the need is not for peer-review in the traditional Western sense. The idiom of Soviet science differs from that elsewhere. The need, in any case, is to find among the small army of people committed to a life in science those who show exceptional promise in teaching and research. That task should not be impossible, given energy and goodwill. Even money should be no great obstacle: £10 million a year net of administration costs would probably help to keep 10,000 Soviet scientists afloat. Is that not a price worth paying for a demonstration that science is, indeed, international? □

Hard times for Polish industrial researchers

- Government science funding overhauled
- Basic research fares well by comparison

Warsaw

POLAND'S industrial researchers, accustomed to guaranteed state subsidies, will next year come face to face with the harsh reality of market economics. With the entire Polish budget for scientific research in the hands of a single powerful committee that is determined to protect basic research from the worst of the spending cuts required as Poland's nascent market economy struggles to find its feet, 1992 will bring unemployment for many industrial scientists.

Since the fall of Poland's Communist regime, the machinery of government science funding has been overhauled in record time. The former separate science budgets of each ministry and the Polish Academy of Sciences have already been rolled together and given to a new state Committee for Scientific Research, set up last spring and chaired by neurophysiologist Witold Karczewski. Whether this rapid progress continues now depends on the Polish parliament's efforts to form a coalition

IMANISHI-KARI AFFAIR

Baltimore resigns

DAVID Baltimore, the embattled president of Rockefeller University, announced early this week that he would resign his position at the end of the month. In a 2 December letter to the Rockefeller board of trustees, Baltimore wrote that the continuing misconduct investigation focused on the 1987 *Cell* paper he co-authored with Tufts University researcher Thereza Imanishi-Kari had "created a climate of unhappiness among some in the University that could not be dispelled". In the last four months, two prominent scientists and sometime critics — Nobel Laureate Gerald Edelman and diabetes researcher Anthony Cerami — have left Rockefeller for other institutions.

"When I accepted the position of President of this institution [in July 1990], I did not anticipate that [the *Cell* paper] matter would become such an extended personal travail for everyone involved," Baltimore wrote. "Trying to govern the University under these conditions has taken a personal toll on me and my family which I can no longer tolerate." He said that he hopes to return to AIDS research at the university, where he will remain a professor. Rockefeller professor Torsten Wiesel will take over as acting president while a search committee looks for a permanent replacement. Christopher Anderson

government, after October's inconclusive elections.

The Sejm, the lower house of the parliament, will try to appoint a prime minister on 5 December. One fear is that the wheeler-dealing may result in the gift of Karczewski's job — which carries ministerial rank — to a career politician lacking Karczewski's expertise in science policy.

Karczewski's committee (known by its Polish abbreviation, KBN) is an unusual hybrid, with two-thirds of its members elected in a poll of some 30,000 active PhD-level researchers, and the remainder appointed by government. All of Poland's researchers, whether in universities, the institutes of the Polish Academy of Sciences or laboratories attached to the health, environment, agriculture or industry ministries, must now look to KBN for their financial support. Given the severe economic recession now gripping the country, many scientists are concerned about their job security. But it is Poland's more than 4,000 industrial scientists who have the most to fear, as KBN is forced to tighten its purse strings.

Karczewski has already had to cut KBN's planned budget for 1991 by more than 30 per cent (from around \$1,000 million) and next year's budget may be even tighter, as Poland's annual inflation rate hovers around 50 per cent and the national budget deficit grows. A new scheme of competitive grants to reward Poland's best researchers has so far suffered the brunt of the cuts — slashed to around \$50 million, about a quarter of the sum planned by KBN. But Karczewski wants to expand this scheme (eventually to consume half of the Polish science budget), and warns that KBN is no longer prepared to pay the basic running costs for institutes ranked poorly in an assessment of all Polish laboratories, completed earlier this year. Many of the lowest-ranking institutes were from the 150 or so laboratories linked to Poland's state-owned industries.

"We should certainly protect basic science," says Karczewski, who points out that most of the institutes of the Academy of Sciences, the stronghold of Polish basic science during the Communist era, scored well in KBN's ranking list. "But we can't protect everybody, and we're not willing to." Karczewski's right-hand man, undersecretary of state Jan Krsysztof Frackowiak, expects the industry ministry to close some 30 industrial laboratories over the coming year, after they are denied

funding by KBN.

Karczewski is quick to dismiss any suggestion that KBN — which is dominated by researchers from academic institutions — is unfairly targeting industrial research for cuts. In a market economy, industrial research should be paid for by its users, he says: if a particular industry requires research denied funding by KBN, then it should finance the work. Frackowiak believes that, under the Communist regime, less than 30 per cent of state-supported industrial research was tailored to meet industry's needs. To illustrate his point, he describes the development by one research group of an advanced car-braking system — a technology that already existed in the West and was not even needed by Poland's low-technology car manufacturers.

Other researchers are more openly critical of the standard of Polish industrial research. Andrzej Ziabicki, from the Academy of Sciences Institute for Fundamental Technological Research in Warsaw, and a leading advocate for rapid reform, dismisses most industrial research institutes as "little more than design shops".

Peter Aldhous

Nature Genetics

Nature, the international journal of science, plans to publish a supplementary journal, *Nature Genetics*, beginning in April 1992. The new journal is intended to cater for the publication of the substantial volume of research expected to flow from present interest in the better understanding of the human genome.

To that end, *Nature Genetics* will consider for publication research articles in human genetics proper, both clinical and otherwise, studies of the genomes of other organisms likely to have a bearing on the human genome and studies in human genetics involving a degree of detail that could not be accommodated in *Nature* on editorial grounds, perhaps because they include detailed genetic maps or nucleotide sequences. In particular, the new journal will include research papers longer than are normally included in *Nature*.

Nature Genetics will aim at the highest editorial standards, but its editorial management will be separate from that of *Nature* except in one respect: the authors of articles submitted to *Nature* but not accepted for publication for lack of space will be offered the opportunity of publication in *Nature Genetics*, often without further recourse to referees. It is expected that most submissions will be directly to *Nature Genetics*.

Nature Genetics will be based in Washington; the Editor is Dr Kevin Davies, previously a member of *Nature*'s biology staff.

Subscription details will be published at a later stage.

New science pressure group

Tokyo

In an unusual move, a powerful pressure group has been formed within Japan's ruling Liberal Democratic Party (LDP) to push for dramatic increases in government spending on research.

The pressure group, called the 'special committee to reinforce maintenance of the basic research base and international research cooperation activity', is chaired by Kishiro Nakamura, a former head of the Science and Technology Agency, and includes a broad range of LDP politicians with strong links to the key science-related ministries and agencies, as well as to the all-powerful Ministry of Finance. Among them are two former ministers of education, Takeo Nishioka and Kosuke Hori, and several 'old boys' from the Ministry of International Trade and Industry (MITI), including the committee secretary, Hitoshi Murai, who used to be deputy director of MITI's Agency of Industrial Science and Technology and is now a vice minister of the Ministry of Finance.

Similar committees have been formed in the past to promote, for example, development of space and energy and overseas development assistance (ODA), but this is the first time such a broad-based political group has been formed to back science. As one government official says, "science and technology policy has tended to be neglected by politicians because there is no money or votes in it." Another, from MITI, describes the formation of the committee as "epoch-making".

A number of factors have conspired to create the right environment for formation of the group. Conditions in Japan's run-down national universities have reached a state of crisis, and powerful academics — in particular, Akito Arima, president of Tokyo University — have been calling for a massive injection of funds to rebuild, re-equip and re-staff the universities. Earlier this year, the Minister of Education, Science and Culture, LDP politician Yutaka Inuoe, visited Tokyo University to see what all the fuss was about and ended up apologizing to Arima for the appalling state of affairs he found.

But it is not just academics who are voicing their concern. Leaders in industry — in particular, the powerful Federation of Economic Organization (Keidanren) — are worried that Japan will soon run short of researchers to drive Japan's growing high-technology industry. In October, Gaishi Hiraiwa, chairman of Keidanren, sent a remarkably frank letter to Jiro Kondo, president of the Science Council of Japan, expressing the federation's concern about the government research system. In the letter, he says that the deteriorating research and education environ-

ment in Japan's universities and national laboratories is causing young people to turn their backs on science and engineering. Japan's goal of building a country based on science and technology is "collapsing at the foundations", the letter concludes. Keidanren calls on the government to double its spending on research to 1 per cent of gross national product (GNP) over the next five years to attract young people back to science.

A third factor is the emergence of 'big science' on the international political scene. Each time the Japanese prime minister meets with President George Bush, he is asked for a contribution to the US Superconducting Super Collider. Problems with the global environment have also helped to make science an international political issue.

The new LDP committee held its first hearing on 6 November with a presentation from Keidanren. This was followed on 12 November with a hearing from Akito Arima and Jun-ichi Nishizawa, president of Tohoku University. On 19 November, six young government researchers explained to the committee the problems they face in Japan's fund-starved government research laboratories. And last week (26 November) it was the turn of representatives of private universities.

Next on the committee's agenda is a hearing early this month on a soon-to-be-released report from the influential Council for Promotion of Administrative Reform. The council, which is composed of

top industrialists and academics, is a powerful advisory body to the prime minister. It has two committees, one of which, headed by Kazuo Inamori, president of Kyocera Corporation, and called the 'Japan within the World' committee, is charged with looking at ODA, the influx of foreign workers to Japan, the global environment problem, and, in another sign of the growing political importance of science, the internationalization of academic research.

The Inamori committee, like Keidanren, is keen to recommend that the government should double its spending on research to 1 per cent of GNP. The Ministry of Finance is opposed to such a recommendation, and there is a heated debate on the final contents of the report. Whatever the outcome, the Inamori report will provide powerful ammunition for the LDP committee. And later this month a committee of the Council of Science and Technology, Japan's top science policy-making body, will also release recommendations for the next five to ten years which are expected to focus attention on problems in the government research sector (*Nature* 350, 544; 18 April 1991).

Armed with all of these reports, the LDP committee will push for removal of the strict ceilings on government spending on research, which have been in place for years. Government officials say the committee will probably make a move early next summer in combination with MITI, the Science and Technology Agency and the Ministry of Education, Science and Culture in time for fiscal 1993 budget requests.

David Swinbanks

SOVIET SCIENCE

Exodus likely to increase

Moscow

SOVIET intellectual life has virtually collapsed as a result of the political disintegration of the Soviet Union and the efforts to switch the economy to market principles at a time of galloping inflation and shortages.

On 20 November, the financing of nearly 80 government ministries and other central bodies was stopped. The central government and the republics have agreed to preserve — in modified form — the foreign ministry and the ministries of the interior, of culture, of railways and of power engineering as well as the independent atomic energy organization and the customs administration.

Many institutions of higher education and industrial research are so short of money that they are having to suspend research programmes and reduce personnel. As a result of the economic crisis and the severance of economic ties, industrial enterprises have no interest in financing projects that do not produce quick returns.

The country's largest research institute of metal-cutting lathes, for example, has an order-book for next year only 15 per cent of normal, and has to cut its staff, according to Pavel Solovyov, the institute's deputy director responsible for research projects.

Moscow State University, which has more than 8,000 professors, instructors and research fellows, almost 5,000 post-graduates and 25,000 students, is on the brink of financial collapse. On 20 November, the staff decided to ask the Russian government to take over the university as an autonomous and self-governing institution. But Viktor Sadovnic, the pro-rector, said that even then, "we shall need international help."

In an appeal to Boris Yeltsin, the Russian president, Moscow students warned that they would stage protest rallies if the Russian government did not take steps to improve social guarantees for students and raise stipends. Many other institutions of higher education had to borrow money

HONG KONG

last month to pay students their stipends, and they are unlikely to be able to repay this money without help.

Last week, Yeltsin signed another decree, under which Russia has taken control of the Soviet Academy of Sciences, together with all its institutes and structures on Russian territory. Unlike the former academy, whose members constituted an elite, the Russian Academy will be a more democratic institution. At the same time, the administrative staff and its institutes and laboratories will be trimmed.

Although Yeltsin said in a special decree that social guarantees should be provided for the employees of academic institutions to cushion the effects of the transition to a market-orientated economy, the scientific community is worried about its future. The Russian budget is limited and proposals for the creation of special funds to finance fundamental research exist only on paper.

There is reason to believe that Soviet scientists may soon begin to leave the country in large numbers. According to Igor Makarov, chief secretary of the former Soviet Academy of Sciences, 564 research fellows of academic institutions have left the Soviet Union this year. Some of them have signed long-term contracts and many may never return. Although this is not a large number by Soviet standards, it is more than double that in 1989. Makarov also said that no foreign scientists are coming to work at Soviet institutions.

Another Soviet academic, Eduard Mirsky, said at a recent press conference that working abroad is now the only way to preserve scientists for science. In any case, the Russian leaders are said to have approved a programme for temporary employment (for terms ranging from 12 months to six years) of Soviet intellectuals at US universities, research centres and corporations on a special visa.

For some time now, Soviet scientists have been going abroad to work at the invitation of developing countries wanting to develop advanced technologies that will allow them to break into world markets. Such invitations have come from Greece, Australia, Egypt and Brazil, but many of them are personal, so the extent of this emigration is unknown, although there is reason to believe that it is growing.

A university in São Paulo, Brazil, is currently negotiating two-year contracts with a group of Soviet scientists. According to José Antonio Pacheco, head of the commission selecting potential candidates, the university is looking for specialists in high technology, particularly superconductivity. And although it has been officially denied that Soviet nuclear scientists have been invited by Iraq and Iran, Academician Alexander Fokin says that a brain-drain in these and other fields connected with military purposes may pose a great threat.

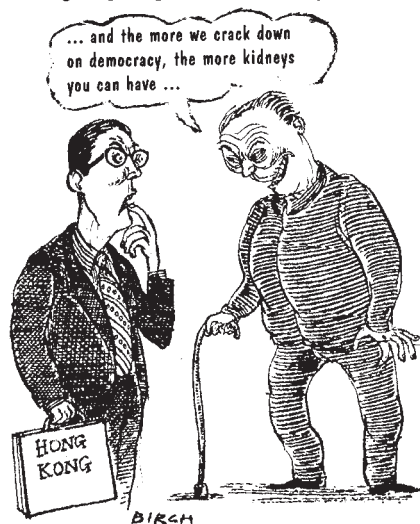
Yuri Kanin

Looking for livers, kidneys

Hong Kong

A SERIOUS shortage of organs for transplants is causing Hong Kong patients to go abroad, some of them to receive organs from executed convicts in China. In response, some Hong Kong doctors are calling for a system in which authorities can remove organs from anyone who dies unless that person had signed a form stating he did not wish to donate his organs.

Tradition among the Chinese who make up 98 per cent of Hong Kong's population demands that bodies be interred whole, and although there are signs that younger, better-educated residents are increasingly willing to pledge their own organs and to



give permission to remove kidneys and other organs from next-of-kin who die suddenly, the general opposition to organ removal has left patients needing transplants with little hope.

Last year in government hospitals, only 55 patients out of more than 1,000 undergoing kidney dialysis received transplants; about 600 patients a year die of kidney failure in Hong Kong. Last month, a team at the University of Hong Kong performed the territory's first two liver transplants, but that success only emphasized the organ shortage: ten patients had died in the two months before the operations because no livers suitable for transplant had become available.

The lack of organs has forced many kidney patients to travel overseas for transplants. The more prosperous head for Australia, North America and Europe, but the less well off frequently opt for China — often with the encouragement of Hong Kong renal specialists.

Doctors in the public medical establishment frown on that option for several reasons, one being that the Chinese transplanted organs are often obtained from executed convicts. Furthermore, Chinese hospitals do little of the tissue typing regarded as essential to reduce the risks of

rejection of transplanted organs, and medical management of patients returning to Hong Kong is complicated by the fact that they often do not bring their complete medical records back with them. Worst of all in the eyes of local doctors and health administrators is the danger that transplant organs will be bought and sold.

Already agents have started to entice local doctors to send transplant patients to a few specific hospitals in China. Earlier this year, a Hong Kong businessman sent a letter to local specialists offering kidney transplants for HK\$100,000 (\$13,000) at the Eastern China Military Region Main Hospital in Nanjing.

That type of commercialization has drawn nearly universal condemnation in Hong Kong. "It's appalling that these things can be allowed to happen," said M. K. Chan, a kidney specialist who has sent patients directly to Chinese hospitals in the past. "It's exactly the situation we don't want to see because you have a middleman charging commission. When we refer patients to China for transplants, the money goes to the hospitals."

The Hong Kong medical community has responded by calling on the government to change its rules on voluntary donations of organs. The territory now has an 'opt-in system', in which individuals who wish to donate their organs in the case of sudden death must obtain and sign a card. Few residents know how to obtain the cards, however, and even when they sign it, under existing legislation the card is not a legal document, and the next-of-kin decides what is done with the body.

Leong Che-hung, a local physician and legislator, and several of his colleagues are calling for an 'opt-out system' similar to that in Singapore, in which authorities would assume that an individual wants to donate his organs unless he signs a form stating otherwise. The government is resisting such an approach, citing its unpopularity with Hong Kong citizens and arguing that medical authorities already approach the next-of-kin of all potential organ donors whether they signed a donor card or not.

Instead of the opt-out system, the government is looking at a number of less drastic options to deal with the organ shortage. It is drafting legislation, which should take effect in a few months, that will prohibit commercial organ trading, and the Health and Welfare Branch is exploring ways of publicizing its donor card scheme and of improving distribution of the cards. Recently, public utilities denied a request to include donor cards with their bills, and now government officials are looking into the legality of distributing the cards with government documents such as water bills.

Peter Gwynne

The future is now (again)

Denver, Colorado

A DECADE after the United States ended its frantic spending on renewable energy research — a binge triggered by the oil crises of the 1970s and killed by the failure of alternative energy sources to live up to their promise — renewable energy has finally come of age.

Wind power now costs as little as conventional electricity sources and is starting to see utility acceptance. Sunlight powers almost 150,000 homes in California. And corn-based ethanol can be found in eight per cent of all US transportation fuels, a figure that will soon rise when new federal emission control laws take effect.

The major difference between now and ten years ago is an increasing emphasis on the bottom line: making useful energy. Without special federal tax credits and subsidies, companies are now building renewable plants that work, not just impressive demonstrations.

The 1970s spending frenzy proved that handing vast sums of money to industry with no strings attached is no way to make progress in research. In 1974, Congress launched a five-year, \$60-million programme to develop working solar energy demonstrations — a programme that consumed \$550 million by the end of 1981, when it was essentially abandoned. At that time, only half of the 287 projects commissioned by the Department of En-

ergy (DOE) were working. Experts had promised that solar heating systems would cost between \$4 and \$8 per square foot by 1979; in fact, the real costs were ten times higher — between \$38 and \$77.

In the area of wind energy, tax credits were given to companies when wind machines were installed, and actually operating the machines earned the companies little more — not a policy designed to produce practical energy supplies.

The lack of results was reflected in the funding. In 1981, the last year of massive spending, DOE's total renewables budget was \$898 million; by 1983 it had been slashed to less than one-third of that.

Now renewable energy research funding is creeping back up. This year DOE expects to spend about \$270 million on renewables — almost twice as much as in 1990. But this time, the money is going to in-house research at DOE laboratories such as the Denver-based National Renewable Energy Laboratory (NREL) and to matching grants to industry. Only companies willing to commit some of their own money to renewable energy get funding.

Lacking reliable federal incentives, research on renewable energy has focused on solid science — genetically engineered bacteria to ferment biomass, new aerodynamics for wind turbine blades, molten salts to convert sunlight into electricity.

But at the same time, researchers are

also showing a new concentration on the balance books. Conversations with NREL's researchers all tend to take the same course: five minutes of science and an hour of policy. No more going for the environmental soft spot; now they argue that renewables make good business sense.

For instance, Norman Hinman runs the laboratory's biofuels programme, where researchers are using biotechnology techniques to create microorganisms that can convert what is essentially kitchen garbage ('cellulose-based biomass' for the purists) into ethanol. Sixty-five per cent of municipal waste could be used to make biofuels, Hinman says, but that will not happen until there is a change in US farm policy. "We could cover the nation's entire transportation needs on biofuels alone," Hinman says, simply by producing biomass on unused farmlands (anything that grows easily, such as grass, would work) and by using the recoverable biomass that now goes into landfills. But the government pays farmers to grow surplus corn crops, which creates a supply of cheap and easily fermentable corn for the biofuel industry, and so the industry has little incentive to re-engineer its technology to use cellulose-based waste, even if the waste is freely available and has to be disposed of anyway.

Hinman's team is working to cut the cost of cellulose-based biofuels until they

The windmill gets a second wind

WHEN one considers the windmill, that quaint low-technology fixture of family farms, "It's not exactly rocket science, is it?" is the phrase that comes to mind. Yet quietly, over the past decade, wind energy has turned out to be renewable energy's strongest contender.

A new airfoil developed by the Denver-based National Renewable Energy Laboratory of the Department of Energy (DOE) managed to boost wind turbine performance by 25 per cent single-handedly by compensating for low winds and the muck that typically builds up on blades. Improvements in the reliability of the gear mechanisms and generators that convert the rotation of the turbine blades into electricity now keep the machines running longer without maintenance. And a new variable-speed transmission design allows turbines to use more of the wind, from breezes to gales. That alone has reduced the cost of wind energy by one-third. In the past decade, these and other technical advances have quadrupled the performance of wind turbines. New turbines can now generate electricity at a cost of around 5 cents

per kilowatt hour, or about the same as coal.

Last month, an Iowa-based electric utility announced that it had joined up with the private US Windpower Inc. to build a \$200-million wind farm in the Midwest United States — the country's windiest region. Expected to total as many as 800 turbines, the facility would generate at least 250 MW of power, enough for 100,000 households. Although the sparsely populated Midwest does not

need the extra power, the consortium partners hope to sell the electricity to neighbouring states with growing energy needs.

In California, which produces 80 per cent of the world's wind energy, windmills now generate 1,600 MW of electricity, enough for the city of San Francisco and totalling about 1.5 per cent of the state's total consumption. The state's biggest utility, Pacific Gas and Electric, announced earlier this year that it would rely on conservation and wind energy alone to handle its increased demand over the next decade.

In Washington, too, wind is back in vogue. Total DOE wind research funding will be about \$30 million next year, twice what it was two years ago, although still less than the \$50 million that DOE was spending yearly at the peak of the renewable energy frenzy of the 1970s. Perhaps the surest sign that wind power has finally come of age is that private industry now spends about \$30 million of its own money on wind research, without federal tax concessions, subsidies or handouts.

C.A.



A nightmare for Don Quixote.

are as cheap as the alternatives. At present, biofuel producers use acids to break down wood waste so that it can be fermented into ethanol, a process that costs about \$1.25 per gallon. But NREL estimates that its bio-engineered enzymes can reduce that cost to about \$.60 by the end of the decade. At that price, biofuels would be no more expensive than gasoline.

In DOE's solar energy programme, too, science must share the stage with policy and economics. It is not enough, for instance, for solar energy to be technically efficient; it must suit the needs of the electric utilities as well. Most power companies arrange their wiring so that each electric substation serves about two dozen houses. That, it turns out, is just about what a small photovoltaic (PV) facility — a half-acre of solar cells — can support. Because the wiring is already in place, NREL technology analysis manager Lynn Coles says that 'grid-connected PV' may be the most painless way for utilities to begin to use solar energy. NREL, along with Pacific Gas and Electric, a California utility, and the Electric Power Research Institute, is planning a trial project.

In September, President Bush officially launched renewable energy's new future by creating NREL — a full-blown national laboratory — out of the old Solar Energy Research Institute. NREL is now starting construction on a new, \$20-million building on land donated by the state of Colorado. But perhaps the surest sign that renewable energy's doldrums are over is NREL's budget — \$125 million, almost exactly its predecessor's peak in 1980, the last time the renewable future looked as bright.

Christopher Anderson

A bright light goes out

In the renewable-energy doldrums of the 1980s, one company managed to keep the solar torch alive. Luz International Ltd was not only the world's largest solar energy research and production company, but by the end of the decade it was generating 95 per cent of the world's utility-scale solar electricity, sending an average of 354 MW (enough to power 140,000 households) into the California electric grid.

Then the whole thing fell apart. Last week, Luz filed for bankruptcy. Although its nine electricity-generating facilities will remain, thanks to binding utility commitments, the construction, research and development and financing companies — Luz's future — are no more.

Ironically, the collapse came just as Luz said its solar energy technology was about to become cost-competitive with conventional energy sources, such as coal. Until it halted construction earlier this year, Luz was building the next generation of solar power plant: a facility, known as SEGS X, in which rows of mirrored channels would focus sunlight on pipes of oil, heating the oil, which in turn would be used to turn water into steam to drive turbines. Luz predicted that SEGS X, planned for completion in 1995, would generate 80 MW at between 6 and 6.5 cents per kWh, somewhere between the cost of natural gas and coal, and less than nuclear plants.

What sank Luz was not bad science, technological hurdles, or bad manage-

ment; it was simply politics. In contrast to the oil, gas, coal and nuclear industries, the solar industry has only one lobbyist in Washington. Every year the federal solar tax credits expire, and every year there is doubt until the last minute on whether that lobbyist will be able to get them reinstated. This year, faced with the possibility that it might not have another year of credits, Luz rushed to finish its latest solar plant in about seven and half months. The crash construction schedule put the project more than \$30 million over budget and wiped out Luz's cash reserves.

Meanwhile, in California, Luz was fighting a losing battle on another front. An error by the state's financial office had mistakenly showed that the company's property tax credits would cost California some \$60 million. Based on that assessment, the governor vetoed a bill renewing the credits. By the time the mistake was rectified (it turned out that each solar plant would actually generate about \$55 million in tax revenues for the state over its lifetime), one of the key investors in SEGS X had dropped out. Other investors and banks soon withdrew as well, citing the political and financial uncertainties.

In July, Luz halted construction on SEGS X and laid off most of its employees. Last week it started liquidation procedures to sell off all but the existing solar plants, which will continue to operate under private ownership. **C.A.**

GERMAN RESEARCH BUDGET

Robbing west to pay east

Bonn

ON 22 November the Bundestag approved a 1992 budget for the German research ministry that is 9.7 per cent more than the ministry's 1991 budget. That increase is illusory, however, because with the unification of Germany, the budget must now be spread over a population that is more than 25 per cent larger than before.

The ruling coalition parties of the Christian Democrats and Liberals settled on a budget of DM9,254 million, a figure that the opposition parties rejected as insufficient. The Social Democrats had demanded a budget of at least DM10,000 million, and one Social Democrat, Emil Schnell, said during the debate on the bill that research will be among the losers in German unification.

Research minister Heinz Riesenhuber has referred to the planned spending as a "budget of solidarity" because it will trim funds in western Germany in order to establish a competitive research infrastruc-

ture in the east.

Out of the DM9,254-million budget, DM1,300 million will go to pay for research in the new *Länder* (states), along with an extra DM300 million from the Ministry of Finance. This figure includes DM585 million to establish new institutes out of the institutes of the Academy of Sciences of the former East Germany; of that, DM416 million is allocated to setting up 'blue-list establishments', new national research laboratories and branches of western institutes.

The budget of the Max Planck Gesellschaft will be increased by 8.9 per cent to a total of DM555 million so that it can establish new institutes in eastern Germany; about 10 per cent of its total budget will flow into the east. And the budget of the Fraunhofer Gesellschaft — the biggest institution of applied research in Germany — will be increased by 83.9 per cent to DM332 million, with much of the increase set aside for research in the

new *Länder*.

When the budget is broken down by research area, space research and technology shows one of the largest increases for the coming year. The space budget will increase by 12.8 per cent, to DM1,737 million, despite the fact that Riesenhuber has retreated from some of Germany's obligations to international space collaborations (see *Nature* 354, 256; 28 November 1991).

Some areas did not fare so well. Particularly hard hit are the 13 national laboratories in the west, which depend on the research ministry for 90 per cent of their budgets and get the rest from the *Länder*. Riesenhuber froze the budget for these laboratories at the 1991 level — DM2,313 million — until the end of 1994. Cuts were made in coal research and technology (down 16 per cent, to DM122.7 million) and nuclear power (down 4 per cent to DM566.9 million). Although researchers in the east may be pleased, it remains to be seen whether research in the west will be able to thrive under these conditions.

Barbara Bachtler

Breach of trust in Sudan

SIR — There has been a serious breach of the principles under which Sudanese biomedical scientists have participated in a long-standing programme of collaborative research on tropical parasitic diseases supported by the US National Institutes of Health (NIH) Programme on International Collaboration on Infectious Disease Research (ICIDR).

Sudanese and US scientists have cooperated in an exemplary way in this research since the beginning of the grant award in 1979. There have been many important jointly authored publications, and opportunities for the growth and development of Sudanese researchers. Recently, however, both the letter and spirit of the NIH ICIDR programme have been abused by administrative actions at Michigan State University, the US counterpart institution (1979–90).

Data and patient samples from our collaborative work were removed without authorization from the laboratory of the former programme director, Professor Jeffrey Williams, by a student whom he had dismissed. A manuscript based on these materials has been published (*Trop. Med. Parasitol.* **42**, 79–81; 1991) and this plagiarism was condoned by university officials. The submission of this manuscript was approved despite our written protest at the highest administrative levels. Neither we nor our US co-investigators were allowed to see the manuscript before submission. The publication includes no acknowledgement of the support of our ICIDR grant, and no credit to the institutions that participated. No authorizations were ever solicited or granted for use of our data or any other intellectual or material contributions. This is totally unacceptable. Our interests as Sudanese scientists have been violated. This contemptuous act must be brought to the attention of the biomedical community at large.

While the NIH ICIDR programme has always been founded on the commonly accepted principles of scientific collaboration, specific provision has been made for the involvement of investigators from developing countries by the following NIH stipulation: "that publications resulting from the collaboration will be coauthored by the foreign scientists, and that the data will be made readily available to the government of the host country at all times (for example NIH, RFA 15 September 1987)". These conditions have not been honoured. Our efforts have been exploited.

Unprincipled violation of international agreements cannot be permitted to go on without public exposure, especially when the interests of tropical medical research scientists in developing countries have

been compromised. Such resources are too scarce to be so mistreated. We believe those responsible should be called to account for their actions.

M. O. A. MALIK (Chairman, Medical Research Council*), MOHAMMED HAG ALI (Director, Medical Research Council), MAMOUN HOMEIDA (Director, Soba University Hospital), HASSAN M. ALI (Dean, Faculty of Pharmacy), HASHIM WARSAMA GHALIB (University of Juba), SUAD M. SULEIMAN (Ministry of Health, Sudan), EL-HADI A. EL-SHEIKH (Khartoum Eye Hospital), OMER ZAYED (University of Khartoum Medical School) BABIKER MAHMOUD (School of Pharmacy)
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Cancer treatments

SIR — Your Munich correspondent's account¹ of the foundation of our new centre for tumour biology is broadly correct, but his description of the centre's scientific aims was less careful. The nondiscriminating equation of alternative cancer treatment with quackery is premature. There is a wide consensus among oncologists that many conventional cancer treatments are inadequate and that new and innovative approaches are desirable. These approaches will necessarily be 'alternative' to the existing ones. Studies of the genetic basis of cancer, that is, insight into the identity and the mechanism of the action of oncogenes and tumour suppressor genes, will provide many opportunities in the years to come.

The first diagnostic applications are already being implemented. It is conceivable that cancer therapies will be designed that specifically exploit the activation of individual proto-oncogenes — for example, the interruption of specific growth factor receptor–ligand interactions or mitogenic signal transduction pathways triggered by activated receptors. Immunological approaches in which the body's defence against tumour cells is exogenously supported might become valuable; for example, antibodies and antibody chimaeras directed against epitopes with enhanced expression on tumour cell surfaces (c-erbB-2 overexpression in breast and ovarian carcinomas) can be produced because of progress in genetic engineering. Recombined receptors may allow the direction of cytotoxic T cells against tumour cells. Plants may harbour valuable compounds that have not been adequately used or tested in oncogene research. The suppression of c-jun/AP-1 activity by curcumin (dietary pigment responsible for the yellow colour of curry) as the basis for the inhibition of PMA-induced tumour

promotion on mouse skin might serve as an example².

"Picking a path for the new clinic"¹ may not be trivial, but will certainly be exciting in times of rapid progress in the understanding of cancer biology. A little benevolence towards this innovative enterprise from a scientific journal would probably not hurt.

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Cover story

SIR — In order that the receipt of individual periodicals issues can be accurately and precisely recorded, it is helpful if all the essential information can be displayed on the outside front cover. When faced with a pile of periodicals to be recorded, it is exasperating for the librarian to have to open up the issue to retrieve essential information. Additionally, the size of print is often far from user-friendly for those of us extracting details.

I have conducted a brief survey using the 115 current periodicals received in the MRC Human Genetics Unit library, looking at the presence or absence of certain essential elements. An 'ideal standard' is the display of the volume number, the year, the part/issue number, the date, the ISSN, and the range of pages that the issue spans: the first four are most important for recording receipt. To take some examples: the *European Journal of Immunology* has no volume number on its front cover — it is on the spine; *Byte* has no volume number nor part number on the front cover — they are on the spine; *Laboratory Practice* has no volume number nor part number on the front cover — it is on the inside; *New Scientist* has no volume number on the outside — it is on the inside; *Science* has no part number on the front cover — it is on the inside (two pages in). *Scientific American* has no volume number nor part number on the front cover — they are on the inside (two pages in). Other, less important, deviations are the display of the ISSN number, date and span of pages.

Without wishing to detract from the freedom of design and individuality that periodicals should be allowed to have, is there any way that periodicals publishers could spare a thought for librarians?

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Leadership needed

SIR — Your recent leading article (*Nature* 353, 285; 1991) in favour of a stronger Non-Proliferation Treaty (NPT) addressed principally the behaviour of Iraq, one of the earliest signatories, yet a country now known to have had a major clandestine programme of nuclear weapons development. But there is a stumbling block to continuation — let alone to a stronger treaty. A Comprehensive Test Ban Treaty (CTBT) is viewed by many non-nuclear-weapon states as a *sine qua non* for preserving the NPT regime, yet the United States and the United Kingdom refuse to commit themselves to near-term pursuit of such an accord. For example, the Bush Administration asserted in 1990 that: "The United States has not identified any further limitations on nuclear testing beyond those now contained in the TTBT [the Threshold Test Ban Treaty, banning all underground weapons tests with yield greater than 150 kilotons] that would be in the US national security interest."

A conference is to be convened in 1995 to decide (by majority vote of all countries that signed the NPT) whether the NPT "shall continue in force indefinitely, or shall be extended for an additional fixed period or periods". One proposal, supported by the United States and the Soviet Union, called for a preparatory meeting (to the NPT extension conference) no earlier than 1993. But Mexico, and like-minded countries pursuing a CTBT first, want a much earlier preparatory meeting. Their point is that non-nuclear-weapon states should not agree to preservation of horizontal non-proliferation (preventing the spread of nuclear weapons technology to new countries) unless the present nuclear-capable states make progress on vertical nonproliferation (preventing the development of more sophisticated nuclear weapons).

Progress in strengthening the NPT will surely be linked to restrictions on nuclear weapons development by the United States and the United Kingdom. The Soviet Union has long stated the need for such restrictions, and has recently announced a new unilateral moratorium on nuclear testing.

If a CTBT is not acceptable in Washington and London, then serious consideration must be given to limitations on nuclear weapon testing that are less stringent than a comprehensive ban. An obvious solution would be multilateral agreement on a new threshold, much lower than the present (bilateral US-USSR) 150-kiloton limit. Consideration of such a new limitation would be a mark of political leadership, desperately

needed if policy for the world is not to be set by those who profit from current styles of nuclear testing and nuclear proliferation.

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Pseudo-dilemma?

SIR — Grünbaum argues¹ that even if we assume that t_0 is a well-defined instant at which the Big Bang singularity occurred, that 'event' cannot "have any cause at all in the Universe" (presumably because backward causation is impossible) nor can it "be the effect of any prior cause" (because time did not exist before t_0). Therefore, the initial cosmological singularity must be uniquely uncaused, and the question of what caused the Universe's origin is therefore a "pseudo-problem".

Unfortunately, Grünbaum's objection is pretty clearly a pseudo-dilemma. For he fails to consider the obvious alternative that the cause of the Big Bang operated at t_0 , simultaneously with the Big Bang. Philosophical discussions of causal directionality routinely treat simultaneous causation, the question being how to distinguish A as the cause and B as the effect when these occur together at the same time. There seems to be no conceptual difficulty in saying that the cause of the origin of the Universe is causally, though not temporally, before the Big Bang and acted simultaneously (or coincidentally) with the origination of the Universe. From the nature of the case involved, the cause must have existed spacelessly and timelessly *sans* the Universe and entered into time at the moment of creation.

But why think that such a cause exists at all? The causal inference is based in the metaphysical intuition that something cannot come out of absolutely nothing. On the theistic hypothesis, the potentiality of the Universe's existence lay in the power of God to create it. On the atheistic hypothesis, there did not exist even the potentiality for the existence of the Universe. But then it seems inconceivable that the Universe should come to be actual if there did not exist any potentiality for its existence. Therefore, it seems that a cause of the Universe must exist.

Of course, as Grünbaum reminds us, it is an empirical question as to whether classical Big Bang cosmogeny is a realistic account of the origin of the Universe. But current doubts about the cold, dark matter model of the formation of the large-scale structure of the Universe do

not call into question the Big Bang itself, nor have alternative models, whether quantum² or plasma³ yet turned out to be convincing. Therefore, it seems to me that, like it or not, currently accepted cosmological theory does lend tangible support to the theistic doctrine of *creatio ex nihilo*.

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Just screening

SIR — It is good that some of the ethical issues in science are being discussed in *Nature*, but the quality of the ethical arguments, or statements, is sometimes deficient.

A recent article (*Nature* 351, 591; 1991) states that "a rule that insurance companies should not seek genetic information about potential policy-holders would be unjust to those free from defect". Why is it unjust for the "healthy" to support the treatment of the sick?

The use of the expression "free of defect" is indirectly to call the genetically sick "defective", which is not the language we should find in *Nature*. A later editorial comment correctly points out that everyone contains many dysfunctional alleles, and all people have some "defects" or "abnormality" (*Nature* 352, 11–14; 1991), yet even that still begs the question why people relatively free from identifiable genetic "abnormality" should pay for the care of the sick.

That view goes against the widely accepted ideas of justice in society. Justice requires society to support the sick, the disadvantaged and all those who need help, because of "accidents" of either nature or nurture. A widely accepted theory of justice would have it that a just society is one whose principles we would all accept if we did not know in advance what position, or state of health, we would have in it¹. It is not unjust for the healthy to support the sick to a reasonable standard of care, but it is unjust to the sick to discriminate against them.

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■ *Nature's* line is that the care of the disadvantaged, genetically or otherwise, is a public responsibility that cannot be met by the regulation of private insurance companies. — Editor, *Nature*.

Pragmatics in the greenhouse

M. J. Grubb, D. G. Victor and C. W. Hope

How can an efficient climate treaty be framed to accommodate continually developing science? It needs to control widely differing sources and sinks flexibly while explicitly distinguishing those which are well understood and quantified from others.

NEGOTIATIONS towards a global framework convention on climate change resume next week in Geneva. Vigorous debates continue both about the specific measures that should be included in the convention, due to be signed at the Earth Summit next June, and about the nature and structure of any assessment and further negotiating processes that the convention may spawn.

Discussions are hampered by the range of greenhouse gases and the wide variety of sources and sinks that are involved. Various response options have been considered at the scientific level, but political debate has focused primarily on CO₂ emissions, particularly from fossil fuels, the largest single contributor to the problem.

Concerned about this focus, the US government has promoted the idea of the 'comprehensive approach' to climate change, by which it is simply meant that all sources and all sinks of all greenhouse gases be controlled as a single 'basket'¹. Any negotiated control levels would be applied to the basket, not to sources and sinks individually. Although many proposals for a greenhouse regime assume that gases will be controlled separately, with initial focus on CO₂, the comprehensive approach seeks to control all greenhouse gases — chlorofluorocarbons (CFCs), methane, nitrous oxide and perhaps even tropospheric ozone precursors — at the same time and on the same basis.

The appeal of this approach is that it provides flexibility by allowing choice on where it is cheapest to control net emissions, thus promising the same climate protection at lower cost. Controlling all sources and sinks also promises the greatest leverage on the greenhouse problem, as no cause of climate change escapes uncontrolled. In principle, the approach can be applied to almost any mechanism to slow global warming, such as national emission targets, greenhouse taxes or tradeable permits. In Washington, the idea has acquired hegemonic status among US government agencies, where it is suggested that the efficiency advantages of a comprehensive approach as compared with the 'irrationality' of constraints on individual gases are such that the administration will not consider any agreement containing individual gas targets.

But many others see the comprehensive approach as a cynical ploy by the United States to gain greenhouse credit for reducing CFC emissions while allowing emissions of CO₂ to rise, and even as a way of preventing any agreement on quantified targets by introducing unmanageable complexity and requirements which, in their simple form, seem impossible to meet. Here we examine these objections, and suggest modifications that could retain many of the benefits while avoiding the biggest difficulties.

General concerns

Doubts about the comprehensive approach spring from many quarters. Developing countries are concerned that by including non-industrial gases (such as methane from agriculture), a comprehensive regime will place more burden on them. Although a broader gas/source coverage could increase the profile of emissions from developing countries, this does not (and should not) imply a greater burden; it may simply broaden the case for international resource transfers as a globally efficient way of tackling the problem. Equity is a different issue from allowing trade-offs between different sources and sinks, and can and should be separated from it. To this extent we concur with the view of the US administration, though we argue below that there are other reasons why most non-industrial sources may have to be excluded.

Another concern is that the comprehensive approach allows the image of action without the substance, by including gases which are already being controlled for other reasons — most notably ozone-depleting CFCs. To prevent double-counting, clear rules are needed on the precedence of reasons for control — though some market-based measures, whether taxes or tradeable emission permits, could ease these difficulties as emissions could be subject to multiple disincentives, reflecting their different environmental impacts.

These are primarily issues of accounting and clarification. But despite its theoretical appeal, the comprehensive approach faces major obstacles arising from the differing characteristics of the sources and sinks involved.

Scientific uncertainties

First, many sources and sinks of green-

house gases remain poorly understood. Of all the anthropogenic emissions that contribute to global warming, only CO₂ from fossil fuels and cement, and most CFCs, may be known to within better than $\pm 5\%$. Emissions of CO₂ from deforestation and other land-use changes are uncertain by $\pm 50\%$, and many sources of methane have uncertainties of up to a factor of two. During the past decade, new sources of methane have been added to the budget, suggesting that the range of sources which will have to be controlled in a greenhouse regime that includes methane is not yet fully settled (see ref. 2). The same is true for nitrous oxide, where the budget has changed dramatically in the past few years, and for tropospheric ozone precursors.

Other uncertainties arise in attempting to estimate and compare the radiative impact of given emissions of different gases. Within the past year, the atmospheric lifetime of methane has been revised upwards by 25% (ref. 3), and recent calculations of the indirect impacts of CFCs have suggested that their overall radiative impact may be substantially less, and more uncertain, than previously believed. The radiative impact of tropospheric ozone and precursor gases such as NO_x and hydrocarbons are extremely uncertain. Furthermore, the radiative impact may depend upon the sources as well as the gas itself — for example, although reforestation absorbs CO₂ it also contributes to changes in albedo and tropospheric water vapour which, in turn, significantly affect climate. The radiative impact of CO₂ from fossil fuels is relatively clear, but even for this process the effective atmospheric residence time is far from certain because of uncertainties in the global carbon cycle. Also, integrating short- and longer-term forcing from different gases into a single index is based upon subjective and somewhat arbitrary choices of 'discount rates'⁷ and time horizons^{5,6}.

In the Montreal Protocol, ozone depletion potentials (ODPs) are used as a measure of the relative ozone-depletion power of the respective gases computed in steady-state by a model⁴. Several global warming potentials (GWPs) have been proposed for use in a greenhouse agreement⁵⁻⁷. But because the chief parameters remain so uncertain⁸, the GWP indices vary and are open to consider-

able dispute and revision.

Obstacles to approximation

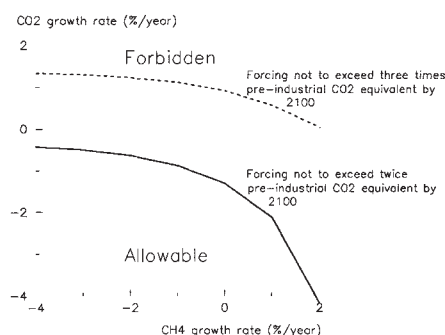
Advocates of the full comprehensive approach recognize some of these uncertainties but argue that suitable approximations can always be made — and that a rough quantitative estimate to bring uncertain sources and sinks into a comprehensive basket is better than none. But the uncertainties are so great, and the implications potentially so large, that agreeing on crude proxy estimates would raise major political difficulties. In addition, relying on such approximations is unrealistic for two key technical reasons: those of monitoring and revision.

Emissions which are made subject to negotiated, quantified controls will require some monitoring. This requirement is more serious than often recognized. For example, both the accuracy and public disclosure of emissions data remain contentious issues in CFC control, and virtually every modern arms-control agreement must contend with sticky monitoring questions — indeed, militarily significant weapons and activities are frequently omitted from agreements precisely because monitoring is too difficult or sensitive. It is unlikely that a climate agreement which imposes significant costs will work if it contains inadequate monitoring provisions, so it must be framed with careful attention to the ability to monitor the sources and sinks it controls. Monitoring can always be improved, but the penalties involved may rise rapidly as well, notably the administrative costs and intrusiveness. Difficulties in monitoring methane emissions from ruminants, which depends upon numbers, species, diet and other aspects of living conditions, suffice to illustrate the point. Even if theoretically possible, it would be administratively very difficult and expensive to include highly uncertain dispersed sources and sinks alongside better defined ones in a single basket.

The second obstacle is that of revision. Significant changes in the estimates of emissions, effective lifetimes or indirect climatic impacts would alter both the absolute and distributive implications of any agreed 'comprehensive' control on greenhouse emissions. Changes would significantly alter the relative costs and benefits of controlling different sources and sinks, perhaps by billions of pounds if control is stringent. If countries had already deployed efforts on the basis of one index, resolving uncertainties and revising the index would be highly contentious and could disrupt the whole system.

Two lists

A single comprehensive basket covering all sources and sinks is not feasible.



Allowable CO₂ and CH₄ growth rates to 2050 with constant emissions thereafter, for different comprehensive bounds. The axes indicate growth rates in the net emissions (emissions less anthropogenically enhanced sinks) for CO₂ and CH₄ from 1990 to 2050, after which net emissions are stabilized to the end of the century. The lines indicate the combinations which result in radiative forcing equivalent to (1) a doubling and (2) a trebling of pre-industrial CO₂ concentrations by 2100. Emissions of CFCs are taken to be as in the London revisions of the Montreal Protocol. CO₂ and CH₄ growth rates are the main variables, and using alternative projections of other gases makes little difference to the results.

How, then, can a greenhouse agreement retain the flexibility of the comprehensive approach? We begin our answer to this question by echoing the obvious: the comprehensive approach is a worthwhile goal, but for reasons discussed above it is not yet fully practicable.

Any substantive greenhouse agreement must start with a limited range of sources and sinks: the most important and the best understood. At present it is probably realistic to expect that only CFCs and fossil fuel CO₂ (also perhaps cement CO₂) are sufficiently monitorable for quantified control levels⁹. These sources could be identified in a climate agreement by listing them — on what we will call the quantified list — just as the Montreal Protocol and subsequent revisions listed controlled substances (the baskets), or as arms-control agreements list controlled weapons. The way would then be open to adopt a comprehensive target and burden-sharing system in a subsequent protocol covering those sources and sinks on the quantified list for which issues of comparability can be adequately resolved, according to an agreed GWP index.

But it is essential that important sources and sinks excluded from the quantified list not be ignored, even in the near term. We propose that these less certain sources and sinks be identified on a second, 'transition' list (our proposal for two lists should not be confused with the two baskets of the Montreal Protocol, which were separated for somewhat different reasons). Though neither their budgets nor their indexes might be sufficiently settled to

be subject to quantified and directly comparable emission controls, those gases could be subject to other forms of controls on a source-by-source basis. The nature of controls would depend on the source/sink involved: some might be subject to quantified proxies (for example, deforestation areas); others to agreements on technically based or 'best-practice' approaches.

Guiding principles could include a commitment to research to reduce the uncertainties associated with sources and sinks on the transition list and an understanding that they should not necessarily be controlled more lightly than quantified sources. This would help to limit any bias to over-control of quantified sources relative to those on the transition list, and reinforce the incentive to improve research. Indeed, the very act of placing a source on the transition list would serve notice that it was likely to be controlled subsequently, even if it were not so immediately.

The initial convention, in establishing the two-list principle, could also specify procedures for dynamically moving gases from the transition to quantified lists, and for introducing new sources and sinks to the transition list. A system of national reporting, subject to national and international review, including commitments to improve data, would be an important complement to this process^{10,12}.

In practice, using just two undifferentiated lists may be too restrictive. For example, for a few sources (such as methane from landfills and coal mines) it is relatively easy to measure accurately the degree of emissions abatement from a given measure (methane capture) although it is very difficult to measure total emissions. Similar issues might apply to some sinks. This might justify a third list, or expansion of the quantified list with separate sub-categories.

But the essence remains the same. A convention needs to recognize both the ultimate desirability of a comprehensive approach, and the immensely different characteristics and requirements of different sources and sinks and the limitations this places on feasible control measures. To this end, a convention should create the principles and structures for classifying sources and sinks into as few categories as possible.

However simplified, it is clear that there will still be a need for frequent renegotiation — both because the scientific and socioeconomic dimensions of climate will change, and because plans must be made for periodic reassessment of the listing of sources and sinks. This might involve periodic negotiation 'rounds' similar to those used by the General Agreement on Tariffs and Trade (GATT). Schemes that put a

premium on flexibility and capacity for renegotiation will have an advantage.

Targets and bounds

With the comprehensive approach (modified as proposed here) one can meaningfully think of an overall goal for controlling the magnitude and rate of change in greenhouse forcing. That goal might even be written into the greenhouse treaty, in addition to the other functions such as creation of the two-list structure. Protocols negotiated subsequently could focus on creating the 'quantified basket' and associated targets and other control mechanisms, including burden-sharing and monitoring arrangements, and could establish other controls for the transition list.

This scheme does not necessarily preclude other emission targets within the initial convention, for within this framework there is an important distinction between individual bounds and the comprehensive emissions basket goal. Proponents of the comprehensive approach are concerned that expressing emission targets for individual gases is logically incompatible with the comprehensive approach, but in reality any comprehensive target implies separate bounds on the maximum possible contributions from the major components.

For example, a comprehensive goal might state an overall level and rate of radiative forcing which it was considered desirable not to exceed at least during the next century; figures of 2 °C absolute change from pre-industrial levels, and a maximum rate of 0.1 °C per decade, have received widespread support¹¹, although all such targets are at present largely arbitrary.

The figure illustrates the implications for permissible trends in CO₂ and methane emissions up to 2050 (if followed by stabilization) of two alternative comprehensive bounds, which result in a radiative forcing by the end of the next century equivalent to respectively a doubling (2×) and a trebling (3×) of pre-industrial CO₂ concentrations. The calculations use a model developed by Cambridge Decision Analysts Ltd as part of EC contract B90/6611/005/63, validated against outputs from the Intergovernmental Panel on Climate Changes. (Details from C. W. H.) This is a simplified model of an extremely complex natural system and serves only to illustrate the nature of the tradeoffs between emissions of different gases.

The IPCC estimated the equilibrium temperature rise from a doubling of equivalent concentrations to be in the range of 1.5–4.5 °C, with a central estimate of 2.5 °C. On the central estimate, the equilibrium temperature changes resulting from the two bounds illustrated — if the atmosphere were somehow then

stabilized at these levels — would be respectively 2.5 and 3.9 °C above pre-industrial levels. Thus, even the more stringent case would probably exceed the targets referred to above.

In both cases, the extra growth in CO₂ that can be bought by agreeing to increasingly stringent controls on methane is subject to rapidly diminishing returns. Considering the more lenient of the two cases, if methane emissions grow at 1% annually, global CO₂ emissions cannot grow at more than 0.5% annually without breaching the 3× bound. If methane emissions can be stabilized at current levels, annual CO₂ growth of just under 1% would be maximum allowed. Even if a 4% annual decline in methane emissions were possible, it would only permit a global growth rate of 1.3% per yr in CO₂ emissions if the 3× comprehensive target is not to be breached. The tougher target cannot be achieved without global CO₂ emissions declining by at least 1% per yr to 2050, irrespective of methane trends. These figures compare with emission growth rates since the mid-1980s averaging about 2% per yr for CO₂ (most mainstream energy projections extend such growth out beyond the middle of the next century), together with steady growth in methane emissions.

The key point is that with fossil CO₂ projected to account for more than two thirds of radiative increases in the twenty-first century⁵, radiative change cannot be reduced substantially without some bounds on CO₂ achieved by emission controls and/or sink enhancements. Nor can exponential growth in methane emissions be allowed to continue. By examining the mixes of different gases which yield equal radiating impact, implied bounds can be derived on the major components for any given comprehensive target. Developing an efficient solution to the immense complexities of full comprehensivity can then be considered in a way analogous to that for solving very complex physical systems — identifying bounds around the 'solution space' and tightening them as research proceeds.

An initial treaty could recognize this and use individual bounds to considerable effect. For example, per-capita CO₂ emissions from developing countries will tend towards the levels in developed countries as the former industrialize. Given the large current disparities in per-capita emissions, it is clear that if the emissions from industrialized countries also continue to rise above current levels, there will be an immense long-term radiative change from CO₂ alone. A bound of stabilizing CO₂ emissions at current levels in industrialized countries, although remote from a fully comprehensive target, is entirely compatible with one. Such bounds might be a crucial

component of a framework convention, establishing it as an exercise which is more than cosmetic, which is an important precursor to the much tougher negotiations which would be involved in negotiating control levels for the quantified list in a protocol.

Conclusions

The potential advantages of the comprehensive approach are clear. But there are concerns about attempting to treat sources and sinks with very different characteristics through a single instrument, and administrative complexities and scientific uncertainties make the fully comprehensive approach unfeasible.

Our two-list proposal is a pragmatic compromise. The creation of quantified and transition lists does not resolve questions about the appropriate degree and distribution of control, but it does hold the promise that negotiated answers on those issues can be practically and efficiently implemented.

The proposal retains the spirit of the comprehensive approach. Climate change is a complex, international problem with many causes, consequences and partial remedies, all of which must be explored in the long transition to a greenhouse-benign economy. This broad view must not be lost in the current attempts to negotiate an initial framework and goals. Quantified controls negotiated in protocols should be as comprehensive as is feasible, which requires a clear list of the sources and sinks whose contribution to climate change can be adequately quantified and monitored. An additional transition list will help to ensure that other sources and sinks — those not well-quantified at present — are not excluded. □

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Towards synthetic self-replication

Constructing chemical systems capable of replicating themselves is an intermediate goal in the reductionist agenda. There is now a little progress to report.

If the properties of living things reflect only the properties of their molecular components, which appears to be the case, it is not sufficient merely to say so, however well founded the evidence for the assertion may be. At some stage, it is also necessary to demonstrate that it is possible to start with a suitable molecular mixture and to follow the replication of some molecular entity in (so to speak) the test-tube. The ideal, of course, would be to reconstruct the circumstances in which the first living things emerged, but that goal is a long way off, if only because so little is known of the original circumstances. But, meanwhile, may there not be simpler systems that demonstrate self-replication?

Indeed, it seems that there are. One of them, just described in print, is nothing less than a scheme for constructing synthetic micelles from polar molecules of sodium octanoate, filling them with the alcohol 1-octanol and using permanganate as an oxidizing agent to form the polar acid. According to Bachmann, Walde, Luisi and Lang (the first three from the ETH at Zurich, the last from the CNRS Institut Charles Sadron at Strasbourg), the micelles multiply in number as the reaction produces more membrane material (*J. Am. Chem. Soc.* **113**, 8204; 1991). The only snag, as the system is set up, is that the micelles become progressively smaller as the reaction proceeds, hardly a fundamental difficulty.

The other approach to self-replication to have been tried seems inevitably to involve molecules capable of forming hydrogen-bonded complexes suggestive in many ways of the behaviour of nucleic acid molecules. Last year, Tjivikua, Ballester and Rebek from the Massachusetts Institute of Technology described a simple chemical reaction between an amino-adenosine and an aromatic ester whose product proved to be a catalyst of the coupling. Moreover, these molecules through hydrogen bonding proved to form dimers which, to the eye of the beholder (or at least the authors) have some of the characteristics of real nucleic acid polymers (*J. Am. Chem. Soc.* **112**, 1,249; 1990). The two molecules of each dimer are presumed to be held together head to toe.

The objectives of both exercises is the simulation of life, or at least of some aspects of it, but readers will not fail to be struck by the diffidence with which this ambition is suggested. Thus Tjivikua *et al.* drop a hint of what they are driving at with

the statement, made delphic by the tense of the verb, that "[t]he ability of nucleic acids to act as templates for self-replication has been unique". At the end they are a little more explicit; they say that their system can, "at best", be regarded as "a primitive sign of life", and continue with the speculation that systems may be designed that will synthesize peptide molecules on a nucleic acid backbone which, they say, would "provide models for events that occurred some time ago".

In reality, those working in this intriguing field have no obligation to be arch about their work, especially when the results are as interesting as these. The auto-catalytic character of the reaction is, of course, crucial. People who mix together hydrogen and chlorine will produce as much HCl as they wish. In the origin of real life, pathways of chemical synthesis involving auto-catalytic steps are likely to be preferred, while it has been found in the past six years or so that some RNA molecules have retained this property, presumed primitive but still useful for the functioning of some cellular machinery.

On this occasion, the reasons why the process is auto-catalytic become apparent. By dissociation, the dimers become a template on which the reactants can conveniently assemble. Each of the two reactants takes up, by hydrogen bonding, the position it would have in a dimer, whereupon the condensation that is the crux of the reaction is easily accomplished. At the very least, this will suggest useful pointers to those who brood about the properties of small RNA molecules involved in editing, splicing and auto-catalysis generally. That in itself is a good reason for not being arch. Indeed, there is much in the belief that the most immediate benefit of attempts to replicate the origin of life will be the understanding it brings of the more complicated processes in real cells.

The tricks that Bachmann *et al.* play with micelles are necessarily more complicated. The self-replication of molecules from a mixture of reactants becomes significant as a pointer to how life may have begun only when it has been shown that the process catalyses itself. But micelles are physical structures of molecules held together by forces of longer range than hydrogen bonding, and whose stability is sensitively determined by the medium in which they are immersed.

The working definition of self-replication is necessarily roundabout. Bachmann

et al. say that the process must involve the multiplication of geometrically closed structures as a consequence of chemical reactions taking place within themselves. They adopt, in short, the principle that one of the essential features of all living things, from cells to mammals, is that they are separated by a well-defined boundary from the medium in which they live, and that their offspring are produced from within themselves as other geometrically closed structures (which may be eggs, not necessarily parental replicas).

For what it is worth, the single-layered micelle system made of sodium octanoate (in which the polar acid group is on the outside of the artificial membrane and immersed in water) is one of several. There are other systems consisting of reversed micelles, formed in organic solvents in which the polar acid groups are on the inside, containing water. This obviously simplifies the process of using more complicated reactions to generate micellar material; one of the systems described uses naturally occurring lipases as a means of controlling the production of octanoate (by the hydrolysis of the glycerol ester).

The results are suggestive, but not compelling, although for a simple reason. As the chemical reactions proceed, the number of micelles in suspension (measured optically) does increase much as the chemical reactions suggest they should. The difficulty, of course, is that each system is fated to have a limited life. In the reverse micelle systems (with the polar groups on the inside), the process must stop when the initial stock of microinjected material has been used up. And the same is bound to happen with the ordinary micelles in which the raw material for new membranes is internally packaged.

Even so, the next steps should be clear. The simple one-layer system cannot allow for the transport of raw materials from the exterior to the interior, which is what happens in real life. For that to happen, all the reactants must be soluble on both sides, which argues for a double layer (as in real life). But it should not be too difficult to construct such a system.

What will that mean? Nothing decisive, of course. Merely that another step in the origin of life has been shown to be feasible. But even reductionists often overlook the propaganda value of these demonstrations. Vitalism, after all, would still be rampant if Wohler had never synthesised urea.

John Maddox

The centre of hypermutation

Ian MacLennan

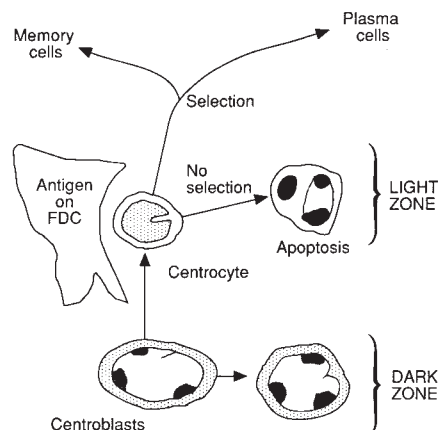
DURING responses to protein-based antigens, the affinity of antibody for the antigen increases markedly. The antigen-combining regions of most of the high-affinity antibodies are encoded by immunoglobulin genes which have acquired somatic mutations¹⁻³, and on page 389 of this issue⁴ Jacob and colleagues report the first direct evidence that these mutations arise within germinal centres. Germinal centres are sites of intense B-cell proliferation and death, and they develop in follicles during T-cell-dependent antibody responses⁵. The new observations confirm the central role played by germinal centres in the maturation of these responses.

Mutations in the genes for the immunoglobulin variable region seem to be introduced by an active site-directed hypermutation process. First, the mutations occur only in the variable-region genes, which encode the antigen-combining sites, and not in the constant-region genes. This difference is unlikely to be due to selection of rare high-affinity mutants, as some mutations found in the variable-region genes of antibody-secreting cells do not increase affinity for antigen. Second, mutations fail to appear in T cell-receptor genes during T-cell responses, or in immunoglobulin variable-region genes during antibody responses not involving B-cell triggering through T cell-derived signals⁶.

The number and complexity of these variable-region-directed mutations increase with the length of time from exposure to antigen and on repeat immunization^{7,8}. During an immune response the variable-region genes of the first antibody-producing cells generated have few if any mutations, but within days antibodies encoded by mutated variable-region genes appear. The possibility that hypermutation occurs at the time of the primary immunoglobulin-gene rearrangement has been considered, but this seems unlikely because virgin B-cell recruitment into T cell-dependent antibody responses is confined to the first few days following exposure to antigen⁹. The appearance of mutations coincides with the time when B cells are proliferating in follicles and particularly with the time when germinal centres are present in the follicles. B-cell proliferation in this site continues long after B-cell activation in extra-follicular areas has ceased¹⁰.

Germinal centres develop within the B-cell follicles of secondary lymphoid tissues during T-cell-dependent antibody responses⁵. The follicles contain a net-

work of follicular dendritic cells which have the capacity to take up antigen and store it on their surface in the form of an antigen-antibody complex¹¹. The B cells that give rise to germinal centres seem to be recruited initially into the antibody response by antigen-driven activation outside follicles. Small numbers of these cells, about three for each follicle,



Outline of the cellular processes that may be taking place in germinal centres. Centroblasts proliferate in the dark zone, and a hypermutation mechanism acts on their immunoglobulin variable-region genes. Centroblasts give rise both to more centroblasts and to centrocetes. Centrocetes migrate to the light zone of the germinal centre, where they may be positively selected if their surface immunoglobulin interacts with antigen held on follicular dendritic cells (FDC). Cells failing to be selected kill themselves by apoptosis. Those that are selected receive differentiation signals which induce them to leave the germinal centre as memory cells or plasma cells.

colonize the follicular dendritic network and enter a phase of rapid exponential growth¹². Within three or four days they fill the network¹³.

At this stage a marked change takes place within the follicle. The proliferating B cells move to one pole of the network, the dark zone of the germinal centre, and cease to express immunoglobulin. The cells, now termed centroblasts, remain in cell cycle but do not increase in number. Half their progeny become centrocetes, which come out of cell cycle and re-express surface immunoglobulin. They pass back into the follicular dendritic cell network, which now fills the light zone of the germinal centre. There is a high death rate among centrocetes, but some leave the germinal centre to become memory B cells¹⁴ or plasma cells¹⁵. Isolated centrocetes on culture spontaneously kill themselves by apoptosis, but they can be prevented from doing so if their surface receptors

for antigen are cross-linked¹⁶. It seems plausible that this reflects a process by which cells that have undergone somatic mutation in their immunoglobulin variable-region genes are selected on the basis of their capacity to be activated by antigen held on follicular dendritic cells (see figure).

In the study of Jacob *et al.*⁴, cells from germinal centres of mice responding to a hapten coupled to protein were identified immunohistologically and removed for analysis of their immunoglobulin variable-region genes. Variable-region genes characteristic of responses to this hapten were amplified using the polymerase chain reaction, and were expressed in bacteria. Analysis of the genes of cells from two germinal centres showed, first, that most B cells within each germinal centre were from a single clone, confirming Kroese's identification of the oligoclonality of germinal centres¹²; next, that the variable-region mutations of most of the identified members of the clone differed from each other, and that these mutations seemed to have been acquired sequentially; and finally, that many of the mutations would not be expected to increase affinity for the hapten.

Germinal centres gradually decrease in size from the time of immunization, so that by three to four weeks centroblasts and centrocetes are no longer apparent. Small numbers of surface immunoglobulin-expressing B-cell blasts can, however, be found in the follicle centre throughout the months of established T-cell-dependent antibody responses. It is unclear if mutations continue to appear in the variable-region genes of these cells.

Similarly, although the study of Jacob *et al.* is of great importance, additional observations comparing the variable-region gene structure of centroblasts with that of centrocetes, and recirculating memory cells with marginal zone memory cells, will be necessary. Analy-

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sis of B cells in T zones at the height of the T-zone response during the first week of antibody responses, and of the B-cell blasts during their initial period of exponential growth in the follicular-dendritic-cell network, also remains to be carried out.

The hypermutation mechanism that acts on variable-region genes remains obscure, but now that cells in which the process is active have been identified

further attempts will be made to seek it out. The mechanism is not only of great theoretical interest but may also be of significance in understanding genetic changes contributing to neoplastic transformation in follicular lymphomas and Burkitt lymphoma. □

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PROTEIN DYNAMICS

A case of a flexible friend

Roger Pain

THE mechanism by which a water-soluble, pore-forming protein inserts itself spontaneously into a non-polar membrane environment raises some intriguing questions. Can, for instance, a protein turn itself inside out? On page 408 of this issue¹, van der Groot *et al.* demonstrate a correlation between the pH dependence of the kinetics of insertion of colicin A into a membrane and the formation of the acid 'molten globule' state of the protein. Given this result, the authors propose that the molten globule state is an intermediate on the pathway to membrane insertion.

Colicin A is a bacterial antibiotic protein that forms voltage-dependent channels in bacterial membranes. The C-terminal domain is responsible for pore formation. Its structure comprises an unusual bundle of ten helices, two of which are buried in the non-polar interior of the domain (*a* in the figure)². In the first phase of membrane insertion following electrostatic interaction with the lipid headgroups, it has been proposed that these two helices partition into the membrane to given an umbrella-like structure (*b* in the figure)³. This idea has received support from the effects of site-directed mutagenesis in the region of the two helices on the cytotoxic activity of the protein⁴. The questions at issue therefore concern the form of any intermediate state on the pathway of this conformational reorganization and the mechanism of destabilizing the native state to provide access to the intermediate.

Van der Groot and colleagues studied the pH dependence of the insertion of the pore-forming domain into a membrane. They obtained first-order rate constants from the time-dependent increase of quenching of tryptophan fluorescence by bromine incorporated at the midpoint of the phospholipids. The apparent pK for insertion of both colicin A and its pore-forming domain is around 4.8.

The authors also examined the pH dependence of the conformation of the

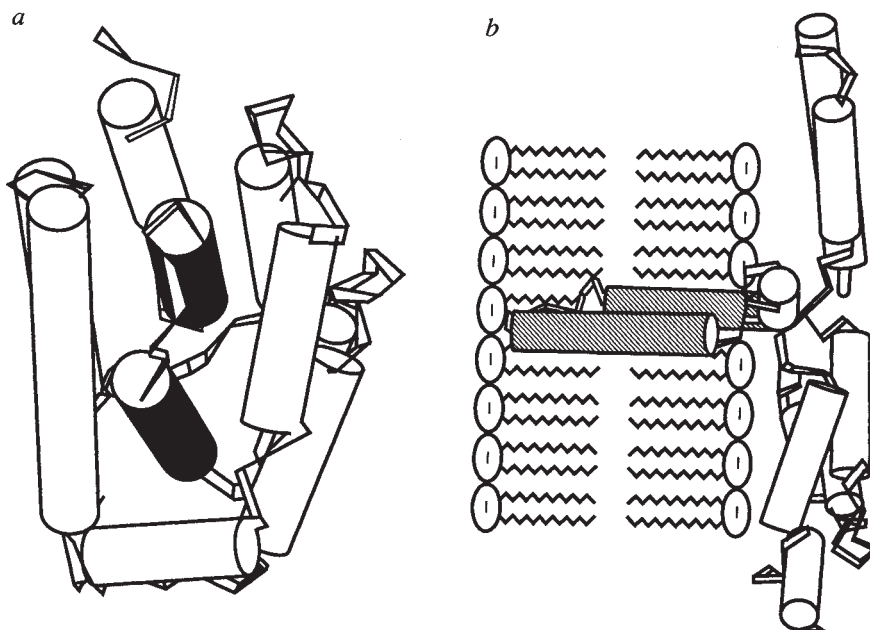
pore-forming domain and found that at pH 2 it exhibits the features characteristic of the molten globule state⁵. The far-ultraviolet circular dichroism spectra show that the secondary structure is similar to that of the native state, while the aromatic residues have lost their asymmetric environment which is responsible for the near-ultraviolet spectrum. The wavelength of maximum fluorescence emission, however, remains at 332 nm, indicating that the tryptophan residues are still largely inaccessible to water and implying that the protein is still in a compact form.

Such a loosened conformation has been shown for many proteins in the presence of acid, alkali or mild denaturant at neutral pH, and a similar but transient species has been demonstrated on the folding pathway of globular proteins⁵. Considerable regions of a pro-

tein have been shown by nuclear magnetic resonance to lack the persistent tertiary interactions characteristic of the native state⁶.

The pK of the transition from native state to molten globule is at pH 2.7, much lower than that for the kinetics of membrane insertion, and van der Groot *et al.* therefore searched for other factors that might indicate a closer correlation. The pH at the surface of a negatively charged surface is lower than in the bulk solution, owing to the electrical surface potential, Ψ_0 . Rather than rely on calculation, the authors obtained Ψ_0 experimentally by measuring binding of a fluorescent naphthalene sulphonate to the vesicle surface. Values of Ψ_0 were used to calculate pH at the interface as a function of bulk pH. When rate constants for membrane insertion of the pore-forming domain were plotted against interface pH, a pK value of around 3 was obtained, which is not very different from that for the molten globule transition. From this correlation, the surface pH is reckoned to bring about a transition to the molten globule state from which partitioning of the helices into the membrane can more readily occur.

Much of the evidence for the early step in protein translocation across mitochondrial membranes is consistent with the involvement of the molten globule⁷. A protein diluted out from denaturant is translocated more rapidly than the initially native protein, whereas in the presence of a stabilizing ligand translocation is inhibited. Clearly, at



a, The pore-forming domain of colicin A, obtained by thermolytic digestion, is composed of ten helices arranged in a three-layer sandwich. The two filled helices are buried in a non-polar environment, whereas the remainder are amphipathic (after ref. 2). *b*, The 'umbrella' model proposed by Lakey *et al.*³ for the insertion intermediate obtained in the absence of a membrane potential. The two non-polar helices have been transferred from the hydrophobic environment shown in (*a*) to the equivalent polarity membrane environment.

neutral pH and in the absence of denaturant, the molten globule state can be populated only to the extent dictated by its free-energy difference from the native state — about 1 molecule in 30, taking the value of $\Delta G = 10 \text{ kJ mol}^{-1}$ measured for β -lactamase⁸.

Similarly, it has to be asked to what extent a protein with a diameter of about 3 nm will be destabilized by the pH at the membrane surface when the bulk pH surrounding most of the protein is some two units higher. It may well be that the interaction of colicin with the membrane lends a helping hand to form a rather than the molten globule state from which partitioning can occur. The concept of the molten globule state in this context is useful in terms less of its structure in bulk solution, and more in terms of its 'moltenness' or 'squidgeability'.

If the interpretation of the correlation demonstrated by van der Groot is sustained, it is likely to have broad applicability. The structure of an insecticidal protein endotoxin⁹ includes a domain consisting of seven helices, the one central helix being buried inside a highly aromatic and non-polar environment provided by the surrounding amphipathic helices. By analogy with the pore-forming domain of colicin A, a model for membrane penetration is proposed that involves the critical insertion of a helical hairpin. Clearly a major structural reorganization must occur if the helices in the putative pore-forming domain are to constitute a pore in the membrane, and an energetically acceptable pathway must be involved.

A more general problem concerns the way in which intrinsic membrane proteins gain access to membranes. What if the kind of low-activation-energy flexibility offered by a molten hydrophobic environment provides a means by which a protein can fold and insert itself into a membrane? There may be something of fundamental significance in the fact that a well-known credit card was advertised as "your flexible friend". □

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Chemistry in a nutshell

Jerry L. Atwood and George W. Gokel

If one trait of modern technology has been the successive miniaturization of components of complex devices, Donald Cram and co-workers¹ have now achieved the ultimate example for the chemical laboratory: the molecular-scale reaction flask. Moreover, they have used this flask to synthesize and study the elusive molecule cyclobutadiene, $(\text{CH})_4$.

Cram has long studied the synthesis and properties of a class of compounds called hemicarcerands, an example of

ment of Cram and co-workers is a milestone in that they have carried out chemical reactions inside hemicarcerands. Moreover, they have now reported the isolation of a much-sought species, cyclobutadiene, within the host molecule. Cyclobutadiene, $(\text{CH})_4$, has surely been synthesized by numerous chemical reactions in the past, but is so unstable that its properties have never been measured⁴. The only spectroscopic studies reported⁵ were made in a frozen

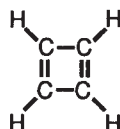
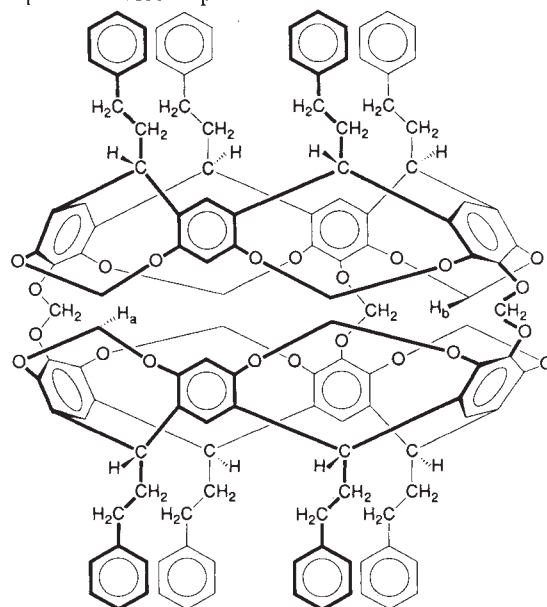


FIG. 1, Right the cavernous hemicarcerand **1** inside which Cram and co-workers have synthesized the elusive cyclobutadiene, $(\text{CH})_4$ (above).



which is shown in Fig. 1. These molecules consist of rigid organic frameworks joined to produce an interior space suitable for the incarceration of small molecules and ions. At the same time, chemists and physicists have been studying the use of solid, porous materials such as zeolites for the entrapment of molecular species. Indeed, impressive results have been obtained with reactions in zeolites², particularly with photochemical reactions³. But zeolites suffer from difficulties associated with the requirements of doing chemistry in the solid state. Cram's hemicarcerand molecules provide molecular entrapment in the solution phase, with associated improvements in available surface area and in the application of physical and chemical methods of characterization.

Against a background of extensive research into the inclusion chemistry of guest molecules within larger host molecules, the new achieve-

ment of Cram and co-workers is a milestone in that they have carried out chemical reactions inside hemicarcerands. Moreover, they have now reported the isolation of a much-sought species, cyclobutadiene, within the host molecule. Cyclobutadiene, $(\text{CH})_4$, has surely been synthesized by numerous chemical reactions in the past, but is so unstable that its properties have never been measured⁴. The only spectroscopic studies reported⁵ were made in a frozen

argon matrix at 8 K. So the stabilization of cyclobutadiene at room temperature within the hemicarcerand is an important advance.

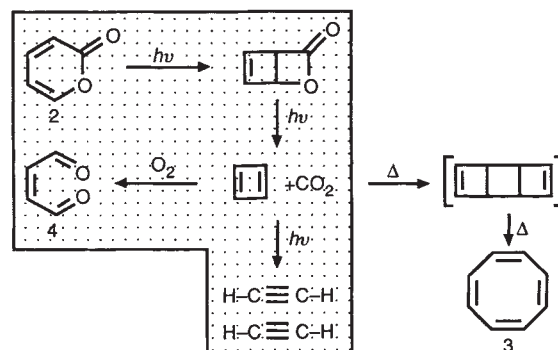


FIG. 2 Inclusion chemistry with a difference: shaded, the reaction scheme used by Cram and co-workers for synthesizing cyclobutadiene, starting with trapped α -pyrone (**2**). Heat (Δ) releases the unstable product, which then dimerizes to form compound **3**, cyclooctatetraene. The addition of O_2 through the hemicarcerand pores oxidizes $(\text{CH})_4$ to trapped malealdehyde **4**. $h\nu$, Irradiation.

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carcaplex compound was isolated by precipitation with hexanes; it was then dissolved in CDCl_3 . Irradiation of the trapped α -pyrone then eliminated CO_2 by a two-step process (Fig. 2) to yield the trapped cyclobutadiene $(\text{CH})_4$. CO_2 is sufficiently small to escape through the hemicarcerand's open pores. Excessive irradiation further photolysed the cyclobutadiene to give acetylene (Fig. 2). The protection offered to the fragile guest molecule was shown by heating the carcaplex, so removing it from the hemicarcerand and allowing it to dimerize and produce free cyclooctatetra-1,3,5,7-ene (3). The authors also showed that it is possible to react the trapped cyclobutadiene with O_2 , which is small enough to enter the hemicarcerand, to produce 1-malealdehyde (1-4).

The molecular reaction vessel will now surely be used to synthesize and study other thermally unstable or highly reactive species. Access to these substances is important in understanding chemical reactions and in testing theoretical calculations.

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Life in the fast lane

Tim Heckman and Chris O'Dea

QUASARS are the most spectacular objects in the Universe: these highly active nuclei of distant galaxies can outshine a thousand normal galaxies each consisting of tens of billions of stars. Of all the amazing things quasars can do, perhaps the most awe-inspiring is their ability to produce titanic 'jets' of radio-emitting plasma, which emanate from the heart of the quasar out to distances that can exceed the dimensions of typical galaxies by an order of magnitude. Such jets are also generic features of radio galaxies — galaxies with active nuclei that are closely related to quasars. Despite nearly two decades of intense investigation, astronomers are still unsure about the physics of jets and their role in the quasar phenomenon. The paper on the jet of the quasar 3C273 by Davis, Unwin and Muxlow on page 374 of this issue¹ is especially interesting, simultaneously providing dramatic support for the popular model, in which the radiating jet fluid travels outward at nearly the speed of light, and placing tight constraints on the same model.

The radio emission from a quasar takes one of two basic forms². In the first, the radio source consists of a very bright core coincident with the quasar itself, a fainter one-sided jet extending out from the core, and even fainter and more extended diffuse emission. In the second, the radio emission consists of a relatively faint central core, two bright and rather symmetric radio lobes strad-

dling the core, and a one-sided jet connecting the core to one of the two radio lobes (see figure). Two of the most puzzling aspects of the entire jet phenomenon are the relationship between the core-dominated and lobe-dominated quasars, and the symmetry of the two radio lobes contrasted with the pronounced asymmetry of the jets. If jets represent the channels along which energy is carried from the core to the radio lobes, why is a jet visible only on one side of the core?

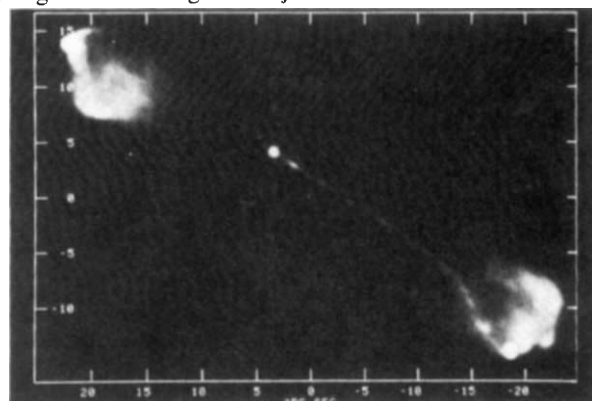
Two primary types of models have been proposed to account for this apparent paradox. The first posits that a quasar intrinsically produces a one-sided jet, and the direction of the jet 'flip-flops' by 180° on a timescale that is longer than the time it takes the jet to travel to a radio lobe, but shorter than the lifetime of the radio lobes³.

The second and more popular model supposes that quasars jets are intrinsically two-sided, but that the radiating plasma moves out along the jets at relativistic velocities, near the speed of light. In this case, the special theory of relativity implies that an observer stationary with respect to the quasar will observe the radiation from the jet plasma to be strongly beamed along the direction of

outflow. Thus, an observer will measure the jet on the near-side of the quasar (the approaching jet) to be brighter than the jet on the far side (the receding jet).

This model is popular for several reasons. First, it naturally explains the phenomenon of superluminal motion — the motion out along a jet of individual knots of radio emission at apparent velocities greater than the speed of light (typically by factors of 5–10). Special relativity predicts that such an optical illusion will occur when an emitting blob moves at near light-speed almost directly towards an observer. Second, the model provides a simple explanation for the Laing–Garrington effect^{4,5}, in which the radio lobe on the same side as the visible jet is seen through a smaller column of magnetized plasma than the radio lobe on the side of the invisible jet. This can be understood if the radio lobes are immersed in a magnetized gaseous halo that surrounds the quasar, and the jet we see is the one pointing towards us (and therefore on the near side of the magnetized halo) because of the relativistic beaming.

Finally, the model is very attractive in a broader sense because it allows seemingly different subclasses of active galactic nuclei to be 'unified' by appealing to distinctions in viewing angle. That is, if the radio emission from jets is strongly forward-beamed, the same quasar will look very different when viewed from different directions. Indeed, it is precisely this effect that is used to unify the core-dominated and lobe-dominated quasars: they may be the same type of object viewed respectively either along or at a substantial angle to a jet outflow axis⁶.



The core, jet and radio lobes of the quasar 3C175 (courtesy of A. H. Bridle).

Despite its successes and its attractive features, the relativistic jet model has still not been subjected to many really critical tests. One clear prediction of the model is that if a sufficiently sensitive radio map is made of a quasar, the much fainter counter-jet (the receding jet) should be detected. Davis, Unwin and Muxlow¹ have made the most sensitive map to date of the best-studied quasar

jet (that of the famous quasar 3C273), and still do not detect any counter-jet at a limiting brightness of 1 part in 5,300 relative to the jet. This limit on the counter-jet brightness is perilously close to the brightness predicted by the model, though the authors emphasize that "the canonical model is not threatened". Clearly, it is important to push to still fainter levels on this quasar.

The second important result is the detection of superluminal motion in the jet at distances of about 400 light years from the core. The relativistic model for one-sided jets requires near-light-speed motion throughout the length of the jet. The controversial case of the radio galaxy 3C120 notwithstanding^{7,8}, superluminal motion is really well documented only on very small scales (tens of light years) at the bright bases of jets. Moreover, the most famous one-sided jet of all — which is associated with the radio galaxy M87 — shows subluminal motion on all scales from light years to thousands of light years from the core^{9,10}.

Although Davis, Unwin and Muxlow hold the current world record in two important categories (lowest limit on counter-jet brightness and greatest distance from the nucleus at which superluminal motion has been detected), we can expect these records to be relatively short-lived. The observational technique of very-long-baseline interferometry (VLBI), with which these results were obtained, is evolving rapidly. The Very-Long Baseline Array (VLBA), currently under construction by the National Radio Astronomy Observatory in the United States, will provide the most powerful dedicated VLBI facility to date. The combination of the VLBA with the existing and future global network of radio telescopes will mean that astronomers can look forward to a decade of exciting discoveries concerning the enigmatic quasars and their jets. □

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CELL CYCLE

Checkpoints and spindles

Paul Nurse

ONE of the more remarkable features of the cell cycle is the extraordinary fidelity of the processes that are necessary for the precise reproduction and segregation of all the components required for successful cell division. Discussing the mechanisms responsible for coordinating these processes, Hartwell and Weinert¹ suggested that a key role is played by 'checkpoints' acting during the cell cycle. This idea has now been cleverly applied to account for the dependence of the completion of mitosis on correct spindle function^{2,3}.

Many events in the cell cycle must be completed in a definite order: for example, chromosome replication has to occur before chromosome segregation, and, as a result, mitosis cannot be correctly carried out unless the earlier event

of S phase has been accomplished. Several mechanisms could account for this temporal control. An early event in a sequence might produce some component or structure that is essential for a subsequent event; such a substrate-product relationship would make the later event dependent upon the earlier one. It would be very difficult to disrupt the timing of events linked in this way, because their dependency is an essential part of the process necessary for progression through the sequence. Temporal order might also be achieved through a timer mechanism operating so that the earlier event occurs after a short time and the later event after a longer time. In this case it would be easy to interfere with the temporal order because blocking the earlier event would not

In the eye of the fly



C. Harris & J. Crum

THE study of invertebrate phototransduction is likely to benefit enormously from new preparations of isolated *Drosophila* photoreceptor cell clusters, which, as reported by C. Zuker and colleagues last month, are amenable to patch-clamp analysis (R. Ranganathan *et al.* *Nature* **354**, 230–232; 1991). Others have also successfully prepared isolated photoreceptors (R. C. Hardie *et al.* *Neuron* **6**, 477–486; 1991). The picture shows a false-colour scanning electron micrograph of one of the roughly 800 photoreceptor cell clusters contained in each compound eye of a *Drosophila* pupa. Each cluster contains eight photoreceptor cell neurons. Ranganathan *et al.* found that the entry of extracellular calcium ions was required for the deactivation of these photoreceptors following exposure to light. What makes the technique especially powerful is that it allows the functional dissection of *Drosophila* phototransduction mutants; screening such mutants revealed that deactivation was defective in the homozygous *inaC* (inactivation-no-afterpotential C) mutant (although excitation appeared normal). Expression of the *inaC* phenotype was dependent on extracellular calcium, suggesting that normal *inaC* activity is calcium-dependent. In this week's *Science*, the same group extend their analysis to show that *inaC* encodes an isoform of protein kinase C (eye-PKC). According to their current model, light-induced activation of the G-protein-coupled rhodopsin results in activation of the *norpA* gene product phospholipase C and production of inositol trisphosphate and diacylglycerol, which in turn promote the influx of extracellular sodium and calcium. The large rise in intracellular calcium activates eye-PKC, which then inhibits phototransduction, perhaps by phosphorylating phospholipase C, the G protein or the light-activated ion channel itself.

Kevin Davies

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RÉSUMÉ

delay execution of the later one. But dependency would still be maintained if a control pathway was included to ensure that the later event would not be undertaken unless the earlier event was completed.

Hartwell and Weinert¹ analysed such control pathways and have proposed the term checkpoint to describe the situation in which an early event is kept under surveillance; if the event is not properly finished, a signal then blocks a later, dependent event. Checkpoints thus enable the cell to monitor progression through the sequence of essential stages of the cell cycle. Unlike the substrate-product dependency, it should be possible to uncouple two sequential events artificially. For example, mutations or chemical treatments that alter one of the steps in the checkpoint control might allow the later event to proceed without completion of the earlier one.

Hoyt *et al.*² and Li and Murray³ have now proposed that a checkpoint control monitors spindle function during mitosis. A microtubular spindle is required for proper segregation of the chromosomes, and impaired spindle function leads to chromosome missegregation. If the spindle is disrupted with microtubular poisons, such as benomyl or nocodazole, progression through mitosis is blocked. The authors have established that a checkpoint control is involved in this dependency of mitosis on spindle function by isolating mutants in budding yeast that complete mitosis and cell division even when the spindle is not functioning properly.

Two different but related procedures for mutant isolation were used. Hoyt *et al.*² treated cells with enough benomyl to knock out spindle function completely. They then screened for mutants that managed to recover after removal of benomyl, arguing that any mutant cells unable to detect or respond to the disrupted spindle function would proceed through mitosis into cell division. Cells dividing in these circumstances would make a poor recovery after benomyl withdrawal and would mostly die because of chromosome missegregation. Three mutants were isolated that continued to divide in the presence of benomyl. The mutants defined two genes, *BUB1* and 2 (budding uninhibited by benzimidazole). A third gene, *BUB3*, identified initially as a multicopy suppressor of *BUB1*, also generated a similar mutant phenotype when deleted.

Li and Murray³ treated cells with a low level of benomyl, which did not completely disrupt spindle function but did slow down spindle assembly. Mutants were isolated that proceeded into cell division at a normal rate during this treatment. These died as a result of chromosome missegregation due to in-

complete spindle assembly. Five mutants isolated in this way defined three genes, *MAD1*, 2 and 3 (mitotic arrest deficient). Because of strain differences, allelism testing between the two sets of mutants is difficult, but the mutants define between four and six genes that might influence the checkpoint control. The fact that only a few mutants have identified so many genes suggests that either the control system is complex and involves many gene products, or the control is indirectly affected by other gene products whose function is primarily concerned with other processes.

These analyses indicate that the mutated genes influence a specific control that checks for proper spindle function before mitosis is allowed to proceed. That spindle malfunction is a checkpoint is demonstrated by the fact that cell cycle mutants leading to other defects in mitotic progression do not complete mitosis and cell division if combined with *bub* mutations. Also, the drop in cell viability of *mad* mutants treated with benomyl occurs only when the mutant cells complete mitosis and is totally suppressed if mitosis cannot take place because of an earlier block in the cell cycle. As expected, treatment of the mutants with benomyl massively increases chromosome losses, and the stability of chromosomes is reduced even in the absence of benomyl. This second result suggests that these gene products do indeed contribute to the fidelity of chromosome transmission during the normal cell cycle.

What is the mechanism by which this checkpoint control prevents further progression through mitosis? One possibility is that it operates by influencing inactivation of the p34 mitotic kinase which brings about mitosis⁴. Cells with persistently high p34 kinase activity cannot proceed from mitosis into interphase until this activity drops^{5,6}. When the mitotic spindle is damaged, either by mutants or microtubular poisons, cells arrest with their p34 kinase activity still raised. But when the spindle is disrupted in the *bub* or *mad* mutants, the p34 kinase activity is not so high and thereafter slowly declines. Perhaps the normal role of the *BUB* and *MAD* gene products is to maintain increased p34 kinase activity should the spindle malfunction, so preventing exit from mitosis until the problem is corrected. In the *bub* and *mad* mutants, the p34 kinase activity cannot be kept up and so cells gradually exit from mitosis and enter interphase.

Little is yet known about the molecular basis of *BUB* and *MAD* gene function. In principle, they could influence any steps in the checkpoint control pathway, including the surveillance of spindle function, the signal generated when the spindle malfunctions, and the response

Hum drum

Will discothèques of the future echo to the beat of the fractal drum, described in *Physical Review Letters* (67, 2974–2977; 1991) by B. Sapoval and colleagues? The drum was designed to test the notion that fractal borders may influence the behaviour of waves, particularly the damping of waves. The drum rim, rather than being the familiar circle, is a sort of cruciform snowflake. To find the drum's resonant frequencies, Sapoval *et al.* excited the drumhead by humming at it with sine tones from a loud speaker. A laser beam skimming over the surface revealed any vibrations. One surprise was to find resonant oscillatory modes that involved only one limb of the 'cross', leaving the rest of the drumhead unmoved. The authors believe the confinement to be a result of competition between wave damping and propagation, the wave's energy being soaked up in the minutiae of the drum's fractal border before it is transmitted through the constricted neck that joins each limb to the main body of the cross.

Acting on actin

CALDESMON is part of a calcium-controlled regulatory system that acts on the thin filaments of smooth muscle. It binds to F-actin, and a binding site close to the N terminus of the actin has been found. Now a second site at the C terminus is reported in two separate papers by R. Crosbie *et al.* and P. Graceffa and A. Jancso (*J. biol. Chem.* 266, 20001–20006 & 20205–20210; 1991). The existence of binding sites for parts of the myosin head at both the N and C termini of the actin subunits suggests that inhibition of contraction may operate by simple steric obstruction.

Beer break

BEER glasses made from toughened glass are hopeless as weapons, say J. P. Shepherd *et al.* in the *British Medical Journal* (303, 1330; 1991). Assaults with fractured pint glasses in the less salubrious of Britain's pubs leave 70 per cent of the victims with permanent scars and cost the state more than £1 million a year in compensation. Shepherd and colleagues tested the impact resistance of several brands of pint glass, both the tankard and the straight variety, and found that toughened straight glasses — even when slightly damaged or scratched — can withstand physical insults far better than the usual annealed straight glasses and tankards; and even when they break, they shatter into myriads of tiny blunt-edged cubes. As well as being more durable, toughened glasses are actually cheaper, and even the most sober of patrons cannot tell the difference. The researchers recommend that magistrates make their use a condition of licensing.

to that signal which prevents mitosis from proceeding. *BUB2* and 3 have been cloned and encode putative proteins of 306 and 341 amino acids, respectively, but these have no significant homology with any previously identified protein or with each other. *MAD2* has also been cloned, and encodes a putative protein of 294 residues which may include three calcium-binding domains. This is of interest given that calcium concentrations may change during mitosis⁷.

The addition of checkpoint controls to those that monitor DNA damage and link mitosis to the completion of S phase (for reviews see refs 1 and 8) suggests that checkpoints are crucial to regulation of cell-cycle progress and in ensuring fidelity during cell reproduction. The concept of checkpoint control may also be pertinent to other developmental processes. Examples of developmental

programmes range from generation of a bacterial spore to generation of a vertebrate embryo, and also consist of sequences of events that need to be executed in a particular order. Crucial points in the programme may be checked, and any errors could delay the process until corrected. □

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The lost continents

Gerald Schubert

THE excitement of discovering a new world, be it another planet or the early Earth, irresistibly draws scientists to the quest. So it is that Galer¹ has revisited the question of what the Earth was like during the early Archaean, 4.5–3.5 billion (10^9) years ago. Did it have oceans, and continents standing above sea level, as at present, or was it entirely different, perhaps without any land above the oceans? Galer concludes that if any continental crust had existed more than 3.8 billion years ago, it would have been submerged to abyssal depths, so that the lack of any sign of such submergence in the mid-Archaean (3.5 billion years ago) suggests that there was relatively little continental crust at the time. Further, the underlying mantle could have been no more than 150 K hotter in the mid-Archaean than at present.

Continental growth

The enigma of how the continental crust has grown has long fascinated geologists, geochemists and geophysicists². It is intimately tied to the physical and chemical evolution of the Earth — the thermal and dynamic states of the mantle and the style of tectonics during the Archaean. Continental growth is so fundamentally complex that its study requires drawing together such diverse topics as isotope geochemistry, geology, seismology and geodynamics. Galer approaches the problem of how the volume of the continents and the temperature of the Earth have changed by exploring the consequences of the geological observation that the freeboard of the continents (the height of the continents above sea level)

has remained approximately constant since the mid-Archaean.

This has been done before but Galer adds a new twist — the possibility that the oceanic crust was thicker in the Archaean than it is at present³. The model he and others use relates the volumes of continents and oceans compensated for their 'buoyancy' to the thermal state of the evolving mantle. A thickened Archaean crust has a dominant influence on this 'isostatic' balance of continents and ocean basins because of the large density difference between crustal and mantle rocks.

Even a model as simple as isostatic balance between continents and oceans requires a host of assumptions if it is to be extended back to the earliest eon of the Earth's history. As formulated by Galer and others, the model assumes that ocean basins of the Archaean were similar in structure to the ocean basins of today. Specifically, it relies on the validity of the plate-tectonic ocean basin, characterized by cold, rigid and dense lithospheric plates that grow from a central spreading ridge and thicken with distance from the rise. But did plate tectonics operate in the early Archaean? And were continents formed by island-arc magmatism at subducting plate margins, the physical process generally believed to characterize post-Archaean continental crustal addition?

Hotspot volcanism and underplating of the lithosphere may have played a greater role in early continental growth than it does today⁴. Perhaps the early Archaean was a time of hotspot tectonics and unusual ocean basins. After all,

upwelling in a vigorously convecting mantle occurs predominantly in the form of mantle plumes; and plate tectonics arises only because of the rigid rheological behaviour of near-surface rocks and affects mantle circulation only through the descending slabs of cold subducted lithosphere. The hotter near-surface rocks of the early Archaean may have been more viscous and less prone to plate tectonic behaviour. Though not necessarily a model for the early Archaean Earth, Venus has no plate tectonics⁵ but it does have volcanic highlands associated with mantle plumes (D. L. Bindshadler, G. Schubert and W. M. Kaula, manuscript submitted, and ref. 6).

Extraterrestrial impacts

Other crucial assumptions that enter the backward extrapolation of isostatic crustal balance models include the constancy of ocean water volume and mean continental crustal thickness with time. The evidence to support these suppositions has been summarized by Galer and others. Nevertheless, the arguments are not conclusive and the validity of these assumptions must be viewed circumspectly. The latest views on the early atmosphere and oceans are in a state of flux because of the uncertain and sometimes catastrophic role played by extraterrestrial impacts in controlling the early atmosphere^{7,8}. The release of volatiles by small impacts during the accretionary phase of the Earth's evolution may have established an early atmosphere and ocean, but a giant impact such as that which formed the Moon would have completely blown off any atmosphere and ocean. Would the Earth develop a substantial atmosphere rapidly after such an event or would a longer-term build-up be required? Indeed, the whole idea of geological catastrophism argues in principle against extrapolation of Earth models back to the early Archaean, but perhaps that view is overly pessimistic.

In addition to questioning the validity of model assumptions about the early Earth, it is also reasonable to look with some scepticism upon the claim that freeboard has been relatively constant (to within 500 m) for the past 3.5 billion years. The constancy of freeboard is well established for the Phanerozoic^{9,10} (the past 560 million years), but it becomes more uncertain as the principle is extended back to the middle Archaean.

If constancy of freeboard, ocean volume, continental crustal thickness and operability of plate tectonics are accepted back to the Archaean, then the last essential uncertainty in Galer's model is the thickness of the oceanic crust. Neither the thickness nor the den-

TRANSCRIPTION

When the CAP fits bent DNA

David M. J. Lilley

sity of the Archaean oceanic crust are known although it is generally believed that the oceanic crust was thicker in the Archaean because it was formed by melting in a hotter mantle. But just how much thicker was it? Galer adopts a theoretical relation between crustal thickness and mantle potential temperature (approximately the temperature at the base of the oceanic lithosphere) derived from isentropic melting beneath a spreading ridge¹¹.

It is this connection that ties the model to mantle temperature and leads Galer to conclude that changes in mantle temperature since the middle Archaean must have been less than about 150 K to accommodate constant freeboard. The constraint on mantle temperature would be relaxed if smaller crustal thicknesses than assumed were associated with higher mantle temperatures. Another compensating effect which is dismissed by Galer is the possibility that the Archaean oceanic crust was significantly denser than the present basaltic oceanic crust because of its komatiitic composition (an increased crustal density would offset the buoyancy of a thicker crust). Galer's rejection of this possibility is based on a prediction of a lack of komatiites in isentropic melting beneath a hotter spreading ridge. Aside from the possibility that the prediction is not accurate, there is the chance that hotspot melting contributed more importantly to the Archaean oceanic crust than did ridge axis melting, in which case the Archaean crust would be more komatiitic in composition and denser than Galer assumes.

Although Galer's specific conclusions about the mantle temperature and continental crustal volume during the Archaean are questionable because of the inevitable assumptions and uncertainties, he has identified an important property, the thickness of the Archaean oceanic crust, which will have to be accounted for in any future models of the evolution of continental crust. □

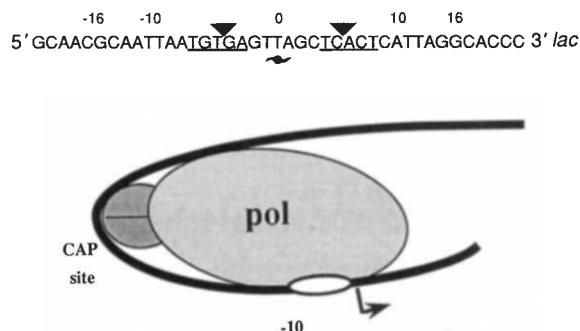
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DNA-BINDING proteins are of immense importance in the control of transcription, from the regulation of bacterial growth to the development of higher organisms¹. At one time it looked as though DNA geometry was not altered very much by complexing with these proteins, give or take some gentle bending or kinking. There was, however, evidence that DNA was strongly bent by a transcriptional activator protein² and now, seven years later, the crystal structure of this complex, determined by Schultz, Shields and Steitz³, confirms that this bend is considerable. Moreover, in a paper published last month, Kerppola and Curran⁴ show that the zipper oncoproteins Fos and Jun also bend their cognate DNA, and the idea of DNA as a passive framework for proteins seems to be giving way to that of a more equal partnership. How then are these distortions of DNA structure translated into biological function?

Our understanding of the mechanics of DNA-protein interactions has steadily increased as structures of DNA-binding proteins have been solved by X-ray crystallography or NMR spectroscopy¹. The helix-turn-helix DNA-binding motif of repressor proteins now has its equivalent in the homeodomain⁵, bearing out the initial prediction that protein α -helices 'read' DNA sequences in the major groove. This means of generating sequence specificity can now be extended to the zinc-finger proteins⁶, and is probably the key to DNA-leucine zipper interactions as well. But trying to summarize this fast-moving subject is akin to photographing an express train head-on — even the principle of helices-in-grooves is no longer universal⁷. The thrust is sustained by Tom Steitz and his group, which has moved on from the structure determination of a prokaryotic transcriptional activator⁸, the catabolite activator protein (CAP), also called cyclic AMP-receptor protein), to solve the structure of its complex when it binds to DNA³.

When carbon sources of energy are restricted, a number of *Escherichia coli* operons, such as *lac* and *gal*, are induced to produce the appropriate enzymes so that alternative sugars can be catabolized. This can be achieved by the bind-

ing of the cAMP-CAP complex to specific sites from 40 to 100 base pairs upstream of the promoter⁹. The complex binds as a dimer to a consensus binding site consisting of TGTGAxxxxxTCA-CA sequences. But these strong binding interactions occur over a region spanning 28–30 base pairs¹⁰, which is much larger than is usually found for repressor proteins. For a relatively small protein (210



CAP protein binds as a complex with cAMP to a series of sites between four and ten helical turns from the transcription start site. Top, sequence of the CAP-binding site for the *lacP1* promoter; the site is located at -61.5 base pairs relative to the start site. The numbering is relative to the site dyad axis. The nucleotide bases of the consensus region are underlined. The position of the dyad is shown, and the large kinks observed in the crystal structure are indicated by arrows. Bottom, wrapping of DNA around the CAP-RNA polymerase complex. This is somewhat oversimplified, as the CAP complex can probably take up more than one position relative to the polymerase, as indicated by the variable spacing. Arrow indicates the startpoint and direction of transcription.

amino acids) to contact a 100-Å length of DNA would require significant bending of the axis around the protein, and sequences outside the TGTGA region could be important in facilitating this distortion¹¹. So part of the selectivity of binding of CAP might come from the ease with which the DNA can accommodate the required bending. The new crystal structure of Schultz *et al.*³ shows that most of the necessary bending is brought about by two discontinuous kinks at the central TpG steps of each of the TGTGA sequences that are related by the dyad axis of the complex (see figure). These kinks involve a loss of base stacking, generating changes in axis trajectory of 51° and 43°.

Sequence recognition by CAP thus has two components. There is the now familiar insertion of a recognition helix of a helix-turn-helix motif from each dimer into the major groove at the TGTGA sequence, where side chains make specific hydrogen bonds with base-pair edges. In addition, there are contacts between 13 other side chains with 11 phosphates per half site, covering a total of 28 base pairs in the complex. As well as making

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favourable contacts, some side chains have a discriminatory role, making unfavourable contacts with mutant sites.

The CAP complex selects for the bendability of its target DNA in two ways. Sequence contacts made at the kinks actually require that the DNA be bent at this point. Also, the sequences further removed from the centre exhibit the kind of variation found in DNA wrapped around histones in the nucleosome¹². Thus at one turn of helix from the centre of the binding site, where the DNA faces the protein, the sequence is A+T rich, providing a compressible minor groove, while half a turn further out the sequence is G+C rich. The total bending brought about by the combination of kinking and bending is about 90°, in good agreement with earlier estimates.

This leaves the most difficult question of how the CAP complex activates transcription at about 20 different promoters in *E. coli*. In this respect, understanding the mechanics of how CAP operates is more difficult than explaining the action of a repressor, which can work simply by sequence-specific binding and occlusion of the promoter. The situation is further complicated by variation in its mode of action at different promoters. First, CAP-binding sites are found at different distances from the promoter¹³. Second, some promoters, such as *lac* and *gal*, are actually overlapping pairs that may be differentially affected. And third, the kinetic steps that are altered vary from promoter to promoter^{14,15}. The general kinetic model for the initiation of transcription involves an initial binding of RNA polymerase to the promoter (binding constant, K_B), followed by a second stage where base pairs in the -10 sequence are opened (rate constant, k_2) to generate the open complex. Further kinetic steps are involved once the ternary complex is formed with the addition of ribonucleotides. Rate enhancements through K_B and/or k_2 are found for different promoters.

So how does CAP binding help these promoters? DNA bending is clearly im-

portant, because the CAP site may be replaced by DNA that is permanently curved owing to repeated oligoadenine tracts, giving promoter activation both *in vivo*¹⁶ and *in vitro*¹⁷. Bent DNA could facilitate local helix opening by releasing mechanical energy¹⁸, but this is probably not the case because unstressed permanently curved DNA can activate transcription and, moreover, the bend is preserved during the formation of the open complex¹⁹.

This leaves us with the promotion of protein-protein contacts. There is a fixed phase relationship along the DNA between the binding of CAP and RNA polymerase²⁰, and the only functional spacings are exactly those found in natural promoters¹³. This suggests that there is intimate contact between the two proteins, in agreement with the observed synergy in binding²⁰ and mutational analysis of CAP which has identified a 'patch' on the protein involved in this contact^{21,22}. The combination of CAP and polymerase generates an even greater bend than CAP alone, in the region of 180°, and this becomes still tighter on formation of the open complex¹⁹ (see figure). Such a large bend in the DNA would permit additional contacts with DNA further upstream²³, and it is possibly the formation of such a wrapped nucleoprotein complex that brings about activation. This still leaves open just how CAP helps in the opening of the *galP1* promoter (where k_2 is affected¹⁵), although it is possible that the DNA loop that is formed is constrained to exert a torque on the -10 region, thereby facilitating its opening. But the final link between CAP binding and gene activation is still not fully understood.

CAP can therefore be regarded as a DNA-bending accessory protein used to achieve the overall nucleoprotein structure. In this it is probably one of a class of important proteins that manipulate DNA structure, including the topoisomerases and resolvases. □

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Casting the stone

BOILERS, water-treatment systems and pipes are often slowly choked by crystalline deposits of calcium sulphate or carbonate precipitated from hard water. Some cunning surface-active molecules have recently been synthesized to counter this effect. They are designed to fit exactly on the growth sites of the crystals, thus blocking their further growth. Treated with such an agent, even a highly supersaturated solution is unable to crystallize.

Daedalus applauds this ingenious development, and is taking it further. DREADCO chemists are treating solutions of hard water with crystallization inhibitors, and then evaporating them until they are almost pure calcium salt with just a little water. The resulting dense, syrupy solution is a sort of 'liquid stone'.

A crystallization inhibitor is a form of molecular protection racket; it will punish any isolated act of defiance. Thus one small crystal appearing in the solution will be arrested on the spot, so to speak, and completely prevented from growing. But mass revolt overwhelms the racket. A lot of small crystals use up all the local inhibitor, and can then grow unchecked. So to set his liquid stone, Daedalus simply adds a pinch of the finely powdered solid. Crystals grow and spread wildly, overpowering the inhibitor everywhere. Depending on the particular calcium salts in solution, the result is an instant slab of marble, limestone, concrete or alabaster.

Modern building technology, which is based on such calcium salts, will be transformed. Instead of struggling with ugly, slow-setting concrete, builders will be able to pour DREADCO's 'Instant Stone' into place, set it, and build on it at once. Classical porticos and facades in the grand manner will be easily formed by simple moulding, and repairs to existing ones could be crystallized monolithically *in situ*. Similarly, sculptors and monumental masons will abandon their hammers and chisels. Instead, they will cast the most elegant and rococo statues and tombstones from Instant Marble, and will be able to go into mass production. Even the humble brick wall will go up with amazing speed, thanks to Instant Mortar.

Clever new composite materials will become possible too. Cardboard, glass fibre or plastic foam soaked in Instant Stone will set to a novel reinforced wallboard or roofing material. Tough, resilient Instant Plaster composites will revolutionize interior decoration. And gangsters who specialize in fitting their rivals with cement boots prior to a short sea voyage, will find their task much simplified.

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Light from seismic waves

SIR — Sonoluminescence (SL) — the production of light by the action of sound waves in liquid — has been observed and studied in the laboratory for more than 50 years. But I believe it has been observed in nature for centuries as earthquake lights (EQLs). EQLs are largely a coseismic occurrence, second only to pre-seismic abnormal animal behaviour for difficulty of reliable documentation and lack of a verifiable explanatory mechanism.

Many explanations for the generation of EQLs exist (see refs 1–3). Most proposed mechanisms require assumptions such as the presence of special minerals, gases or organisms, or unverified physical conditions in the fault zone. All have difficulty explaining the persistent reports of EQLs at distances of up to hundreds of kilometres from the earthquake, and at sea or in association with large bodies of both fresh and salt water or wetlands.

I propose that at least some EQLs are not the product of high strain accumulation or shear rupture dynamics in fault zones, but rather result from molecular reactions in water that has been strongly shaken by the compressional (*P*) waves produced by the earthquake. A seismic *P* wave is simply an earthquake-generated sound wave in a solid or liquid; hence if a *P* wave induces light emission from liquid, it is a situation entirely analogous to SL as generated in the chemist's laboratory.

SL is a remarkable consequence of acoustic cavitation in liquids irradiated by sound waves^{4,5}. For it to occur, a cavity or bubble must be created in the liquid continuum, then rapidly compressed. The process adiabatically heats the trapped gas or vapour sufficiently to dissociate molecules. On recombination or return to the ground state, photons are emitted. Once a cavity or bubble is formed, two types of SL are possible: 'stable', in which the bubble resonates and incrementally grows, usually in a standing wave field; and 'transient', in which the bubble expands and implodes all within one cycle of a standing or travelling sound wave. A travelling *P* wave should be an efficient stimulus of transient cavitation, although long trains or strong-motion *P* waves may induce stable cavitation SL as well.

The observed SL spectrum in water has a peak at 310 nm (in the ultraviolet), arising from the return to the ground state of the excited hydroxyl radical (OH); there is also a poorly understood continuum throughout the visible waveband. A pure water SL spectrum will appear blue to bluish-white, but the presence of dissolved salts or other im-

purities can appreciably alter the basic aqueous spectrum⁶, so that yellow or red may predominate.

SL has been generated in the laboratory with ultrasonic pressure amplitudes of 1–2 bar (0.1–0.2 MPa)⁴, corresponding roughly to an energy density in the ambient fluid of 10–20 erg cm⁻³. Whether SL is a viable mechanism for EQLs hinges on the question of whether it is reasonable that *P* waves of sub-audible frequency can supply this energy density in water.

The density of the kinetic energy, *e* (per unit volume), induced in the transmission medium by one cycle of an advancing seismic wavefront is a standard result in seismology⁷ and is given by

$$e = 2\pi^2\rho(A_0/\tau_0)^2$$

where ρ is the density of the medium and A_0 and τ_0 are the displacement amplitude and period respectively of the seismic wave. Conservatively estimated values for A_0 of 1–10 cm and τ_0 of 0.1–1.0 s may be obtained from the strong ground motion recordings of earthquakes. This yields *P*-wave energy densities in water of roughly 500–2,000 erg cm⁻³ at 10–1 Hz and pressure differentials of 1.3–2.7 bar. Thus, seismic *P* waves are capable of supplying pressure changes and energy densities that exceed the laboratory values that induce SL. Within the water volume irradiated by *P* waves, EQLs would arise as the integrated light flux from many SL cavitation bursts, all loosely synchronized by the *P*-wave dilational half-cycles.

In the laboratory, SL produces an illuminance of $\sim 10^{-8}$ lumen cm⁻² (ref. 8), which is visible to the dark-adapted eye. Thus, to reproduce an illuminance equivalent to moonlight of $\sim 10^{-4}$ lumen cm⁻², as reported for EQLs in Japan⁹, $\sim 10^4$ ultrasonic SL bursts are required in the laboratory. For the much larger *P*-wave cavitation events (with bubble radius in the centimetre rather than micrometre range), the same illuminance could arise from a single event. To illuminate a landscape to moonlight brightness from at least several kilometres distance, 10^2 – 10^4 individual SL *P*-wave bursts would be sufficient. A *P*-wave with a dilational half-cycle wavelength of ~ 1 km is certainly cap-

able of spawning such an SL field.

Reports of EQLs for large nocturnal earthquakes are not ubiquitous, but neither are they extremely rare. Selected characteristics relevant to the SL hypothesis that appear in the EQL literature are: (1) distinct blue to bluish-white EQLs reported from coastal Japan¹⁰ and Hawaii²; (2) extensive EQLs from an onshore alluvial setting¹¹; (3) numerous accounts of EQLs sighted offshore from California¹, Mexico¹¹ and other coastal zones; and (4) well-defined, blue and yellow spherical lights in tsunami wavecrests¹². (Luminescent organisms may be another source of luminescence in tsunami wavecrests¹³, but I do not believe that they would create well-defined spheres of light.)

Natural SL is perhaps not the only mechanism that produces EQLs, but it is able to explain a wide range of the existing reports. The hypothesis predicts that (1) EQLs should not be confined to the immediate fault rupture zone; (2) bodies of water must be present, although it is possible that saturated soil can sustain SL; (3) EQLs are essentially a coseismic phenomenon, which in the absence of strong foreshocks or aftershocks should not be observed before or more than several minutes after the earthquake; and (4) the EQL spectrum should contain a prominent hydroxyl peak at 310 nm (and for sea water⁶ a sodium peak at 589 nm). Hence the SL-EQL hypothesis can be tested by means of spectrographic analysis, although obtaining an EQL spectrum will not be a trivial undertaking.

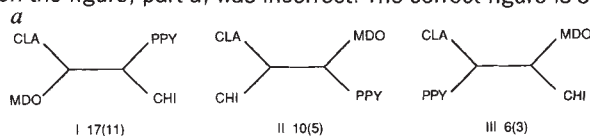
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Correction

In the letter from M. Allard *et al.* (*Nature* **353**, 610: 1991) "Tests for rodent polyphyly", the lettering on the figure, part a, was incorrect. The correct figure is shown below. □



From science fiction to reality?

SIR — The existence of a planet in orbit around a neutron star, reported by Bailes *et al.*¹, was actually predicted^{2,3} by the science-fiction writer Poul Anderson in a novel first published in 1958. Anderson also predicted that a neutron star would possess “The most powerful bloody magnetic field ever noticed around any heavenly body” (ref. 3, p. 78), though his estimate of 10^4 gauss for the surface field strength was much too low (10^{12} gauss for most neutron stars). Anderson argued that a neutron star must have a strong magnetic field, because the field strength should be proportional to the neutron star angular velocity, which should be enormous due to conservation of angular momentum during collapse of a stellar core. Anderson’s physics is essentially correct. (Conservation of flux and angular momentum imply the proportionality of magnetic field strength and angular velocity.) Anderson’s analysis was in advance of astronomical opinion. In a Science Fact article⁴ (in the same issue of *Astounding Science Fiction* as Anderson’s novel), the astronomer Alastair Cameron wrote “the remnant left behind [a supernova explosion] is a white dwarf star.”

As proposed by Anderson, the neutron star’s planetary companion would have “a smaller mass and radius than Earth” (ref. 3, p. 143), whereas the planet discovered by Bailes *et al.* has a most probable mass of about $10 M_E$, where M_E is the mass of the Earth. Anderson makes the interesting suggestion that the planetary progenitor actually had a “mass greater than Jupiter” and “an extremely big orbit” but had its hydrogen atmosphere blown away by the supernova explosion (leaving only the nickel-iron core). The planet lost angular momentum in the process and its orbital radius became much smaller. Such a mechanism for generating an approximately Earth-mass planet does not seem to have been considered by Bailes *et al.* Angular momentum being carried away by the blown-off atmosphere could solve the binding energy problem^{1,5}: unless a planet loses angular momentum, it will become unbound if its star loses more than half its mass in less than one orbital period.

Bailes *et al.* point out that even if a mechanism could be found to keep a planet bound during a supernova explo-

sion, one would expect the planet’s orbital eccentricity to be large, whereas the planet they discovered has an almost circular orbit. Anderson does not discuss this eccentricity problem, though he does mention two physical processes that might be relevant. First, he notes that once the outer layers of hydrogen are removed, the core would “revert to normal density, which would be a pretty spectacular catastrophe in itself” (ref. 3, p. 143). Indeed it would. Hubbard *et al.*⁶ have constructed models for the interiors of Jupiter and Saturn assuming both have a core mass of about $20 M_E$. Thus an estimate of the energy released by blowing off the outer atmosphere of Jupiter and reducing its mass to Saturn’s is $P_J V_J - P_S V_S$, where V_J and V_S are respectively the volumes of the cores of Jupiter and Saturn, and P_J and P_S are respectively the average pressures in the cores, obtained by a linear interpolation between the pressures at the centre and the surface of the cores, given in Table 4 of ref. 6. The energy released is 3×10^{41} erg, which is enough to accelerate about $7 M_E$ (7 per cent of Saturn’s total mass) to Saturn’s escape velocity.

The supernova ejecta would be expected to blow back gas emitted toward the star, so the net impulse from this density change would be toward the star. But the most efficient way to reduce eccentricity is via an impulse directed tangential to the orbit (ref. 7, p. 349) at apastron. Friction between residual supernova ejecta and the planet was Anderson’s mechanism of reducing the semimajor axis. This would also reduce eccentricity⁷, but it seems by an insufficient amount. It is nevertheless suggestive that the mass of the Bailes *et al.* planet has a mass roughly equal to the core masses of Jupiter and Saturn⁶.

Do these correct but qualitative predictions entitle Poul Anderson to any scientific credit? Perhaps pulsars with planets should be called ‘Poulsars’.

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Actin tracks

SIR — Theriot and Mitchison¹ have tracked actin in the lamella of moving fish epidermal keratocytes (FEK) using a new photoactivatable actin probe. They observed that the labelled actin filaments remained stationary with respect to the substratum in both locomoting and stationary cells. This interesting result may, however, represent the behaviour of only a subset of the actin microfilaments.

Centripetal motion of fluorescently labelled actin has been observed in non-locomoting fibroblasts² or growth cones³. Theriot and Mitchison suggest that their failure to observe similar movement of actin could be due to an absence in keratocytes of control mechanisms present in fibroblasts and growth cones. But an alternative interpretation is to suppose that there are two populations of actin filaments in the lamella, a small stable population and a larger population undergoing rapid turnover. With photobleaching, a bleached spot would rapidly disappear because of turnover of the unstable filaments, and the stable actin would not be seen against the fluorescent background. With photoactivatable actin, however, one would see the opposite result: the unstable population would quickly fade while even a few stable filaments would remain visible against the dark background. The latter approach thus would tend to emphasize the most stable filaments, especially after longer time periods.

This hypothesis would reconcile some observations which we (D.F.K. and E.L.E.) have made with those in ref. 1. Previously we attempted to track the movements of rhodamine labelled actin filaments in the lamellae of locomoting FEK by observing a photobleached spot at room temperature². We were unsuccessful, in part due to the short lifetime of the bleached spot (5–8 seconds). Using VEDIC microscopy, however, we have observed impressive cytoplasmic waves in stationary and locomoting FEK. There is good evidence that such waves reflect movement of actin³. As in nerve growth cones, the waves move at high velocity with respect to the substrate while the cell remains essentially stationary. The waves retract toward the nucleus upon addition of cytochalasin D, leaving the lamella essentially empty of actin filaments, except for a band at the leading edge and a few coarse filaments. Waves then reappear on washing out cytochalasin D (ref. 4). Our results thus suggest that at least a fraction of actin filaments may undergo constant centripetal flow in FEK.

We suspect that the stable actin filaments seen with the photoactivatable probe represent the ventral cortical

Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. They need not arise out of anything published in *Nature*. In any case, priority will be given to letters of fewer than 500 words and five references. □

cytoskeleton. Cortical filaments, which are linked to the substrate (for example via integrins or other adhesion molecules) might be the most stable. Further, such filaments would not be expected to move with respect to the substrate.

More work remains to be done to determine whether there are different populations of actin filaments distinguishable by their stability or their mobility relative to the cell or substrate. The existence of these hypothetical subpopulations of actin filaments is clearly compatible with functions of myosin in addition to those that are considered by Theriot and Mitchison, and with models of cell motility other than the nucleation-release model.

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Theriot and Mitchison reply — The possibility that two (or more) populations of actin exist in the lamellipodium with very different dynamics was certainly of concern to us. We decided against discussing that possibility mainly because of lack of space, and because we saw nothing in our results that required the existence of a second population of filaments. Specifically, we never saw a broadening of the activated bar, even at 5- or 10-second time points, which would be strongly predicted if a moving population of filaments coexisted with the stationary population. In addition, we saw no reproducible deviation of the intensity versus time turnover curves from exponential, even at early time points. If a large population of filaments existed with shorter average lifetimes, we should have seen biphasic decays. But of course these arguments are certainly far from conclusive.

In our experiments¹, the cells were cooled to 15 °C, in part because preliminary experiments at 23–25 °C had indicated very short bar half-lives, probably closer to 5 or 10 seconds than the 25-second half-lives we saw at 15 °C. This temperature difference might in part explain the quantitative difference in our results.

It seems, though, that even if a second

population of filaments exist, whose average lifetime is of the order of 5 seconds rather than 25 seconds, the basic requirements of our nucleation-release model that the filaments be short relative to the dimensions of the lamellipodium and that they be both polymerizing and depolymerizing throughout the entire structure are indicated even more strongly. The spatial movements of this hypothetical second network are of course a separate and more interesting issue.

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Channel control

SIR — Hartzell *et al.*¹ concluded that a membrane-delimited pathway for modulation of calcium channels by the stimulatory guanine nucleotide-binding protein G_s does not function in amphibian and mammalian cardiomyocytes. Because their conclusion regarding mammalian cardiomyocytes differs from ours² and others^{3,4}, we believe that a pivotal experiment presented by Hartzell *et al.*¹ in their Fig. 2d may have been misinterpreted.

Isoprenaline (ISO, 1 μ M) quickly increased rat myocyte calcium current (I_{Ca}) from about 1.5 to about 2.85 nA, and dialysis of 5 mM adenosine 3',5'-(Rp)-phosphothioate (Rp-cAMP-S, an inhibitor of protein kinase A (PKA)) abolished the stimulation over the next 20 min. The authors drew a straight line between the pre-ISO values (about 1.5 nA) and the late Rp-cAMP-S values (about 0.9 nA), and attributed the difference to I_{Ca} rundown. They said: "Assuming that I_{Ca} rundown occurred at a constant rate during an experiment, 1 μ M ISO increased I_{Ca} in rat 127% $\pm$ 13% and 5 mM Rp-cAMP-S decreased the ISO-stimulated current 117% $\pm$ 9% (slightly below the control level, $n=7$)".

The wording is ambiguous, but Fig. 2d shows that Rp-cAMP-S after isoprenaline restores I_{Ca} to (rundown-compensated) control amplitude. With regard to rundown compensation, we feel that the linear interpolation between pre-isoprenaline and late Rp-cAMP-S I_{Ca} amplitudes (20 min elapsed time; Fig. 2d in ref. 1) is an unsupported assumption. However, giving the authors the benefit of the doubt, the reduction of isoprenaline-stimulated I_{Ca} to control level by Rp-cAMP-S actually supports our earlier conclusion.

Our reasoning is based on two series of unpublished experiments related to our studies² on guinea pig ventricular

cardiomyocytes. In the first of these (dual-pipette method), pressure-assisted test dialysis of control solution containing 1 mM Rp-cAMP-S was preceded by dialysis with control solution (5 mM ATP, 1 mM GTP, and so on; see ref. 2 for technical details). Rp-cAMP-S reduced basal (control) I_{Ca} by 27 and 32% within 2 min ($n=2$). In the second series (single-pipette method), I_{Ca} after 10 min dialysis with control solution containing Rp-cAMP-S was 25–40% ($n=4$) smaller than after 10-min control dialysis ($n=8$).

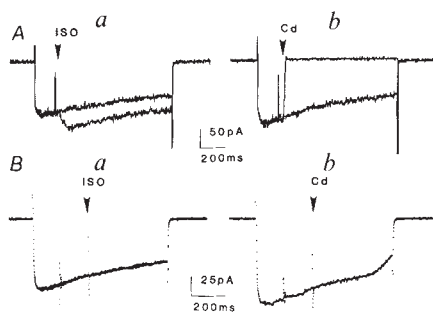
Thus, it appears that Rp-cAMP-S, like heat-stable inhibitor protein and regulatory subunit of PKA⁵, blocks both isoprenaline-induced and basal phosphorylation and therefore should reduce isoprenaline-enhanced I_{Ca} below its basal pre-isoprenaline level. Because an analogous reduction was not observed by Hartzell *et al.*¹, it must have been offset by concomitant stimulation. With PKA blocked, the latter stimulation can be attributed to a non-cytoplasmic, GTP-dependent component of stimulatory isoprenaline action mediated by G_s (refs 2, 6).

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SIR — Because they did not observe a fast response to β -adrenergic stimulation in adult guinea-pig cardiac myocytes as we reported⁷, Hartzell *et al.*¹ claim that the response does not exist. In our view, this is a version of the 'all swans are white' argument; the figure (over page) shows that isoproterenol (Iso) applied as a concentration jump during the slowly declining phase of the voltage-sensitive Ca^{2+} channel current produced a rapid increase in current ($126 \pm 11\%$, mean $\pm$ s.d., $n=14$). Both rate and magnitude of the increase were concentration-dependent¹. The fast response has been observed in at least 53 experiments, 23 of which we described in ref. 7, and we are certain of its existence. The fast response was not observed under two conditions: when cells were leaky as the outward tail currents in Fig. 2f of ref. 1 indicate; and when the block of Ca^{2+} channel current by Cd^{2+} was delayed (see figure), unlike the rapid block that we required as a control¹. Under these same two conditions the slow response, although attenuated, persisted. Thus, the fast response is not as robust as the slow response and perhaps this is why Hartzell *et al.* missed it. Another factor may be methodological differences. The perfusion system of Hartzell *et al.* is different from ours and the delay in response to acetylcholine is much longer

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Rapid increase of Ca²⁺ channel currents produced by concentration jumps of isoproterenol (Iso, 100 nM) (Aa, arrow) and rapid blockade produced by Cd²⁺ (1.0 mM) (Ab, arrow). Recording and solution conditions as described in ref. 1. Switching transients are produced by the solenoid valve of the concentration clamp. When the concentration change was slow, the rapid response was absent (Ba) and the Cd²⁺ blockade occurred after a long delay (Bb).

(compare their Fig. 1b with Fig. 1C of ref. 7). They do not seem to have used the rapidity of Cd²⁺ block as a control and their Fig. 2e shows that the 'jog' of Iso actually decreases Ca²⁺ channel current. Furthermore, their assumptions regarding Ca²⁺ channel-current rundown in the presence of Rp-cAMP-S are probably invalid; Ca²⁺ currents should fall below, not return to, control levels. The fast response has also been demonstrated in adult guinea pig cardiac myocytes using a different experimental approach⁸.

With regard to mechanism, we favoured the direct G protein effect but did not exclude a rapid cytoplasmic pathway⁷. The reasons for our choice were that neither Forskolin, IBMX (1) nor 8Br-cAMP produced the rapid response. Subsequently we have added Wiptide, a peptide inhibitor of PKA, to our patch pipettes and greatly reduced the slow response without reducing the rapid response ($n=7$).

Hartzell *et al.* claim that the conditions in intact cells under which direct G protein effects on Ca²⁺ channel currents have been demonstrated² were non-physiological. However, the same direct effects have also been observed under solution conditions virtually identical to their own⁶. Second, Hartzell *et al.* make the novel proposal that Ca²⁺ channels might be under direct G-protein control in the absence of agonist. They appear unaware of the fact that this proposal, unmodified, overthrows the fundamental concept of G proteins as signal transducers. We recently proposed⁹ a modification that might obviate their dilemma.

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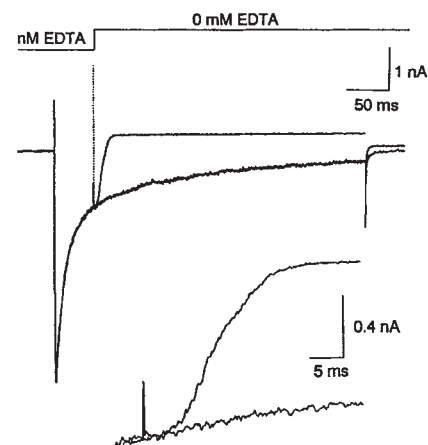
HARTZELL *ET AL.* REPLY — The purpose of our paper¹ was to test the hypothesis⁷ that a direct G-protein pathway is necessary to explain the effects of sympathetic nerve stimulation on heart. We do not conclude that direct G-protein mechanisms do not exist. Rather, we document that under physiological conditions a fast pathway, presumed to be due to a direct effect of G proteins, is absent on cardiac Ca channels. This, together with the observation that the inotropic response to sympathetic stimulation can be explained entirely by cAMP-dependent phosphorylation, implies that the direct pathway is neither a proven physiological mechanism nor needed to explain the physiology. A direct pathway may exist, but it is of little import for the cardiac response to sympathetic nerves. This position is supported by the very small size (<50 pA) of the fast response reported by others⁷.

We initially examined whether the fast response was a general phenomenon. We developed a new perfusion system¹ to test the question in frog, rat and guinea-pig hearts. We switch between two flowing streams, whereas Yatani and Brown switch from a static bath to a rapidly moving one. The sudden flow past the cell could possibly introduce uncontrolled variables. Quantitative data for the Wiptide experiments are not provided, and in any case would not exclude a flow effect. Our perfusion system appears sufficiently rapid. The cell responds to [K]⁺ changes in <30 ms, the time courses of our acetylcholine response¹ and their carbamylcholine response⁷ are the same, and the effects of (–)BAY K 8644 occur within 150–300 ms. Moreover, the figure shows that the Na current through Ca channels in the presence of EDTA is blocked within 30 ms of washing out the EDTA.

Yatani and Brown's figure Bb is perplexing. It is unclear why Cd blocked so slowly. Furthermore, the data in Aa seem different from their previous results⁷. The fast response here develops with a $\tau \approx 40$ ms, compared to 150 ± 35 ms in their previous report. Moreover, Iso does not affect I_{Ba} inactivation, whereas they previously found that it slowed inactivation.

The suggestion that we missed a fast response because our cells are leaky is fallacious. We show non-leak-subtracted traces. The 10–50-pA outward current at 0 mV is not non-specific leak; it is residual K current not blocked by Cs. Holding current was $< \pm 5$ pA. In any event, a role for leak is difficult to rationalize: direct G-protein effects are observed in artificial lipid bilayers¹⁰.

Our suggestion that Ca channels may be affected by G proteins in the absence of β -adrenergic agonists does not question the role of G proteins in signal



Block of I_{Ca} carried by Na ions in frog cardiac myocyte by rapid perfusion switch from a Ringer solution containing 5 mM EDTA, 0 Ca to the same solution lacking EDTA with 3.6 mM Mg. Inset, region of the switch enlarged.

transduction, it merely points out that G proteins may perform other functions.

The unpublished data of Pelzer *et al.* that basal I_{Ca} is partly inhibited by R_p-cAMP-S suggest that the Ca channel is partially phosphorylated in the absence of β -agonists. If the cAMP system is activated under basal conditions, it is likely that the direct pathway would also be activated, because the direct pathway was said to be more sensitive to β -agonists⁷. If this is true, sympathetic stimulation would be unlikely to stimulate further the direct pathway and it would not participate in the physiological response to sympathetic stimulation. Furthermore, the physiological significance of the fast pathway would be minimal in the presence of basal sympathetic tone.

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The physics of solitude

Mark Walker

Uncertainty: The Life and Science of Werner Heisenberg. By David C. Cassidy. W. H. Freeman: 1991. Pp.669. £23.95, \$29.95.

WERNER Heisenberg (1901–1976) is one of the most controversial, most ambivalent and most important figures in the history of modern science. He is universally honoured for his contribution to modern physics, including quantum mechanics and his uncertainty principle, but his conduct under National Socialism, and in particular his work for the German uranium project, have provoked both condemnation and apologia. The literature on Heisenberg is polarized and politicized and ranges from demonization to hagiography.

This is the definitive biography of Heisenberg, an intensely psychological book in which Cassidy skilfully interweaves the necessary portions of the histories of modern Germany and physics to create a tapestry of Heisenberg's life and science. There are a few technical portions, inaccessible to the laity, but, even for the nonspecialist, Cassidy provides a good explanation of quantum mechanics and Heisenberg's contribution to it. By being both objective and critical, he has made Heisenberg into a much more human and sympathetic figure than have previous biographers. The biography is based on Cassidy's prodigious research in primary sources and his mastery of the extensive secondary literature. If that were not enough, he has managed to unearth some hitherto unknown information, so that even the specialist will find the book enlightening.

Cassidy really aims to explore and explain the darkest and most fascinating side of the story: how and why Heisenberg entered into a Faustian pact with National Socialism. Cassidy provides an excellent account of Heisenberg's crucial experiences as a teenager during the short-lived Bavarian Soviet Republic and his participation in the bloody counter-revolution. Heisenberg's uncompromising opposition to communism began then and stayed with him for the rest of his life. Most importantly, it clouded his judgement once the Second World War became a war of ideologies between fascist Germany and the Soviet Union.

Perhaps of even more influence on Heisenberg, however, was his involvement in the Weimar youth movement.

Heisenberg became the leader and father figure for a group of younger boys, and Cassidy persuasively argues that the values Heisenberg adopted at this time influenced his conduct under



Why did Heisenberg form a pact with National Socialism?

Hitler's regime. Heisenberg had accepted full, all-encompassing and lifelong responsibility for his charges in the youth movement, and once he had become an established, successful scientist, he felt the same way towards his students and younger colleagues. Thus Cassidy provides an explanation for why Heisenberg never left Germany, and why he made certain compromises with the National Socialist regime in exchange for the opportunity to train the next generation.

When Hitler came to power and the new National Socialist government began to purge and harass Jewish scientists, Heisenberg, by now a professor at Leipzig University, reacted in exactly the same way as his respected senior colleagues Max von Laue and Max Planck: they tried to persuade the dismissed physicists to remain in Germany and worked behind the scenes to rescind the dismissals. Once it became clear that the dismissals would stand, they quickly tried to fill the vacant positions. Cassidy points out that although this tactic might have made professional sense, it also reinforced and supported the racist National Socialist policies: preserving

physics at institutions where Jewish scientists had been fired would support the arguments that the Third Reich was not incompatible with science and might play into the hands of those who argued that the Jews were not needed; participating in finding a replacement for someone who had been unethically dismissed, or who had resigned in protest, could be seen as a tacit acceptance of the grounds for dismissal or a denial of the legitimacy of that protest.

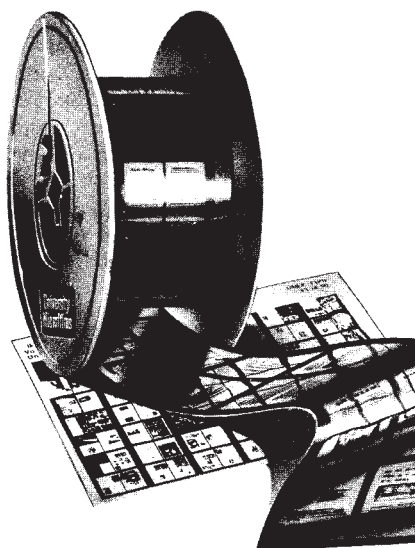
Heisenberg's own relationship with National Socialism is ambiguous and opaque. He never joined the National Socialist German Workers' Party or any other National Socialist organization, but he did hold high scientific positions throughout the Third Reich and performed required duties without complaint. On a personal level, he disapproved of what he regarded as Nazi excesses, yet he never broke with the regime and continued to pursue his science. One of the most important aspects of Cassidy's biography is his demonstration that Heisenberg's attitude towards National Socialism changed over time.

Heisenberg was attacked repeatedly by Nazi colleagues as a "white Jew". His response was to work within the system, in this case through the SS, in order to win his political rehabilitation. But as Cassidy points out, perhaps most important for our understanding of Heisenberg's conduct is that he began to take the political attacks on modern physics as being increasingly personal. Heisenberg came to regard his own survival in Nazi Germany as tantamount to the survival of decent physics, and the continued survival of some elements of decent physics provided the grounds for his own continuation of the struggle. Thus Heisenberg and his colleagues sought to demonstrate the utility, and in particular the military value, of their science to the National Socialist hierarchy. They were also willing to strike a humiliating bargain: in return for political rehabilitation and more generous support, Heisenberg and his fellow physicists agreed to expunge Jewish names from their science.

It was war and the discovery of nuclear fission that finally gave Heisenberg and his colleagues the opportunity to prove themselves. In the polarized post-war myths about Heisenberg and the 'German atomic bomb', he and his colleagues are usually portrayed as being incompetent, or as having resisted Hitler by denying him nuclear weapons. Neither extreme is true, and as one who has written about the history of the German uranium project, I could not agree more

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with Cassidy when he states that: "... there is nothing to support the notion that Heisenberg actually hindered the project in any way to keep an explosive out of Hitler's hands or even that he himself had that much control of the situation."

The postwar period was also an unhappy one for Heisenberg. His own apologia together with Samuel Goudsmit's polemics damaged his relationship with many of his foreign colleagues.

German physics, for which he had struggled so long, was now eclipsed, and his own research was not as successful or as well received as it had been.

Uncertainty is an exquisite book, especially because it neither demonizes nor canonizes a great scientist and troubled man. □

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Love and the lonely cybernaut

Christopher Anderson

Virtual Reality. By Howard Rheingold. Simon and Schuster/Secker and Warburg: 1991. Pp. 415. \$22.95, £16.99.

WHAT does it say about this world when people find such creative ways to escape it? In the sixties and seventies it was psychedelic drugs. The eighties were video games and computer networks. And now, combining both the people and techniques of three decades of escapism, comes virtual reality, the nineties' way to holiday in your own head.

Virtual Reality is computer-groupie Rheingold's tour through a fringe research world of hackers and visionaries who build and explore simulated ('virtual') worlds, seeking to get ever closer to what could pass for the real thing. From the laboratories of NASA (the National Aeronautics and Space Administration) to a dusty back porch where the ageing inventor of "Sensorama" keeps a rusting prototype of his coin-fed "experience theater", Rheingold traces the evolution of what has in less than a decade become a multi-million dollar industry of high-tech illusion.

In *Brave New World*, Aldous Huxley's characters went to the "feelies" and held metal balls to get that great 3D-and-beyond sensation. Now, virtual reality's 'cybernauts' wear video helmets and strap themselves into "data gloves". Either way the aim is the same: a machine-made out-of-body experience. The difference is that virtual reality apparently works.

At a trade show two years ago, telecommunications managers nearly rioted to get into "Reality Built for Two", a virtual-reality demonstration project built by the pioneers VPL Research. And for good reason — the lucky few that made it in got to wear an "EyePhone" and a "DataGlove" and chase each other's computer-generated images around a virtual world of

geometric shapes and rooms. To the outside observer, they were people in helmets gesticulating to themselves. But to the participants, who could see nothing but what the computer showed



Science Photo Library

Virtual reality, or surreality? Certainly not the real thing.

them, they were as close to being inside a computer as it was possible to be. Watching someone experience virtual reality, writes Rheingold, "is a bit like watching them hallucinate."

In fairness, virtual reality promises more than just the world's coolest video games. Military types are developing flight simulators based on virtual reality, and architects are already 'walking' clients through virtual-reality floor plans of proposed buildings. Surgeons may soon wander around inside a virtual representation of their patients before they 'suit up' and cut. And researchers may find that being inside a virtual rendering of their data is the best way to explore it.

For the moment, however, virtual reality's insatiable appetite for computer horsepower keeps it out of reach of all but a handful of dedicated laboratories. Even there, virtual images are still cartoonish and jerky, owing mostly to the tremendous processing overheads of creating real-time 3D graphics. In computer-graphics terms, processing power is measured in 'polygons per second' — all virtual images, from din-

ner plates to human shapes, are made up of combinations of straight lines and boxes. The more lines a machine can draw, the closer it can approximate to the subtle curves of nature. Today's graphics computers can generate several-hundred-thousand polygons per second. Impressive as that may be, no one is going to confuse those images with the outside world. "Reality", one expert estimates, "is 80 million polygons per second."

This does not stop Rheingold from speculating about what life will be like when the computers catch up. He devotes a chapter to "teledildonics", which one is to believe will be something like intimate one-on-one video teleconferences where the distant participants wear electric suits layered with sensors and transducers to simulate every caress. (A dirty limerick attributed to the computer pioneer John von Neumann suggests that such outfits should be easy to clean.) Just what a computer virus might mean in this context is left to our imagination.

Rheingold is at his best when chronicling the exploits of virtual reality's colourful pioneers. Jaron Lanier, president of VPL Research, is a dreadlocked "bear of a man" who lives in darkened rooms full of musical instruments and sells futuristic body sensors to both NASA and the toy company Mattel. And John Walker, a young man "with the kind of haircut you can still get at a barbershop for \$5" who wears a pen-protector full of pens in the breast pocket, is the president of Autodesk Inc., a billion-dollar drafting-and-design software company on the verge of "going virtual".

Virtual Reality is written by an unabashed convert and fan. At his worst, Rheingold tends to romp through the world of technology in breathless first-person fashion: "The hand that floated in the virtual world was more than a hand. It was me." But in those passages where he manages to suppress his own awe and marvel for long enough to describe what the researchers in the field are actually doing, he generally gets his facts right, and his prose is clear enough to convey what the virtual-reality cast is

really like. They are, in a word, eccentric (one should be rightly sceptical of any movement that has Timothy Leary, guru to the LSD generation, as a spokesman). But they seem to be on to something quite interesting indeed. □

Christopher Anderson is a Nature staff reporter.

Group therapy

R. Bruce Martin

The Biological Chemistry of the Elements: The Inorganic Chemistry of Life.

By J. J. R. Fraústo da Silva and R. J. P. Williams. Clarendon: 1991. Pp. 561. £60, \$75.

THE cover of this volume features the theme of life, with Adam handing Eve a tile depicting the first element in the periodic table. Tiles for the biologically important alkali and alkaline earth metals, sodium, potassium, magnesium and calcium, nine transition metals, and the nonmetals phosphorus, sulphur, selenium, chlorine and bromine and iodine, form the backdrop to the scene. With the addition of silicon, the picture celebrates the elements emphasized in this rich and refreshing book. (No IUPAC-inflicted 1–18 group-numbering used here.)

Robert J. P. Williams, Royal Society Napier Research Professor at the University of Oxford, is arguably the world's most knowledgeable person on the comparative chemistry of the elements. While still a student he developed the well-known Irving–Williams stability series. At least a third of a century ago he pioneered the field of bioinorganic chemistry, well before the area became *de rigueur* in the United States. Because Williams's numerous publications cut a wide swath, it is useful to have this book where his breadth of knowledge, viewpoints and insights reside conveniently in one place.

In the preface, the authors state their intent:

The strict environmental conditions that are required today by living organisms also point to the necessity of strict internal control and regulation of these processes, and this again means time and space organization of the biological substances, both large and small, and of their reactions. Much of this control is the now well-known classical treatment of the organic chemistry of life but, diverging from the classical tradition, we shall regard it in this book as the interplay of not just the great number of small and very large organic molecules but also of a group of about 25 chemical elements, free as ions, combined as complexes, or as precipitates, organized in biological space and time. Most of these elements are classified as 'inorganic'. We shall go on to insist that a proper appreciation of living systems and their evolution cannot be obtained without reference to these elements.

Thus not only carbon but many elements are judged central to life.

To carry out this promise, as well as for easy reading, the profusely illustrated book is divided into three parts, 22 chapters and sections within chapters. The seven chapters in the general first part cover uptake, speciation and compartmentalization of the elements in biology; kinetics and control; energy and hydrogen biochemistry; and the roles of biological macromolecules of all kinds. This broad overview is followed in part two by detailed discussions of individual elements.

The spirit of the 12 chapters in the second part may be captured by quoting the opening of the chapter on zinc:

At the beginning of each chapter on a given metal in biology we should have in mind the question as to why it has been selected for its particular tasks. In the present case the question itself has two parts: (1) why zinc rather than another metal ion; and (2) why use a metal ion at all since zinc is only an acid catalyst comparable to the proton.

The first two parts of the book prepare the reader for the integration in the third part, which is composed of an opening chapter containing an interesting discussion of biological shape and the integration of the functions of different elements; a climactic chapter on the elements in homeostasis, morphogenesis and evolution; and a final chapter on what we, as successors to Adam and Eve, are doing with the elements in our Garden of Eden, and the consequences that this might have.

In addition to being a general resource, this engaging book provides an excellent text for an advanced course and should have much to teach both students and teachers. A small fault is that only short bibliographies (often mentioning lengthy reviews) at the end of each chapter have been listed, and that no specific references are given in any section within the chapter. For this omission, the authors offer a weak apology at the end of the preface.

Some years ago Williams noted that, as a group, physiologists were more aware of the importance of inorganic substances in biology than most biochemists. Recently, the imbalance has been lessening and many inorganic chemists have discovered biology. All these groups will gain by studying this book, in which their disparate viewpoints are so admirably synthesized. □

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Addendum

The full title of the book reviewed by John Durant on page 714 of the 24 October issue of *Nature* is: *Persuading Science: The Art of Scientific Rhetoric*. □

■ Two other books on virtual reality have recently been published: *Cyberspace: First Steps*, edited by Michael Benedikt, contains essays on the theoretical and conceptual issues involved in the design, use and effects of virtual environments (MIT Press, \$29.94, £24.95); and *Mirror Worlds, On: The Day Software Puts the Universe in a Shoebox... How it Will Happen and What It Will Mean* by David Gelernter (Oxford University Press, \$24.95). Those who find the language of virtual reality impenetrable will find relief with *Technobabble*, in which John A. Barry examines the new computer lexicon from an etymological, historical and anecdotal perspective (MIT Press, \$27, £19.95).

Variations on a theme?

James Mallet

The Development and Evolution of Butterfly Wing Patterns. By H. Frederik Nijhout. *Smithsonian Institution Press*: 1991. Pp. 297. \$45 (hbk), \$20 (pbk).

How does one-dimensional DNA guide three-dimensional development? Colour patterns, which develop in only two dimensions, are simplified systems that could provide insights into this riddle. So it is perhaps surprising that virtually no biologists study manipulable pattern systems such as butterfly wings.

Nijhout has been the exception since 1978 when he outlined a general hypothesis of how butterfly wing patterns are formed. He proposed that many colour patterns could be generated by a series of related, or 'homologous' foci on the midlines of wing cells. These might act as sources and sinks of diffusible substances that regulate pigment production in the scale cells. Nijhout amplifies the hypothesis in this book by showing that the elements of a "nymphalid ground-plan", similar to the pattern of *Argynnis* or *Speyeria* (fritillaries), can act as foci for both simple patterns (such as those of *Heliconius*) and complex ones (such as those of the undersides of *Charaxes*). Nijhout is well known for his elegant experiments on *Precis* eyespot patterns, which, with morphological analyses of normal butterflies, aberrations and pattern genetics, form the support for his hypothesis.

After reading the book I am even more convinced that the evolution of butterfly wing patterns has few constraints. Nonhomologous pattern elements align within and between wing surfaces to produce perfect leaf mimicry in *Kallima*; patterns may be complex for camouflage, or simple for mimicry or sexual signalling, with often both present on the same butterfly, each on a different wing surface; and when one colour pattern will not do, polymorphisms or seasonally variable patterns evolve. Because anything can happen, it is difficult to find testable homologies for all patterns, yet this is precisely what Nijhout attempts.

Well, not quite. Nijhout points out that "ripple patterns" — rhythmic repeats such as those on *Urania* moths — are never exactly the same on left and right wings. By contrast, most other patterns are symmetrical, and are assumed to be generated by interactions between the nymphalid ground-plan and wing veins. No other hypotheses are discussed in the book. For example, the

possibility that *Heliconius* evolved their unusually simplified mimetic patterns by a non-ground-plan innovation is not considered by Nijhout, whereas in 1978 he thought that "colour fields" such as those in *Heliconius* demanded a separate explanation. Nor is Atuhiro Sibatani's alternative hypothesis for eyespot development mentioned, although the relevant paper (1980) is cited by Nijhout in a section on homeotic patterns.

Even if the ground-plan is accepted, Nijhout seems to deduce homologies in the book by guesswork. For *Charaxes* and some papilionids, Nijhout guesses that the alignment of a pair of underside bands with a single upperside band indicates homology: the upperside band must then be a composite of the two

familiar? Although not referenced, Richard Goldschmidt is famous for proposing the same idea in 1945 on the basis of a similar nymphalid ground-plan. Nijhout is even more radical, suggesting that in the müllerian mimics *H. erato* and *H. melpomene*, even the genes that control the colour patterns may be homologous. I am cited erroneously as agreeing with this genetic homology between *Heliconius* mimics; in my paper I actually discuss homologies between races 'within' rather than 'between' species. The forewing bands of *H. erato* and *H. melpomene* seem to me to have about as little in common genetically as might possibly be imagined for such nearly identical colour patterns. Any similarities that do exist between other genes

may be due to simple inheritance of simple mimetic patterns in both species, requiring similar gene action. Crosses between related *Heliconius* species cause a breakdown in pattern, suggesting that clarity of intraspecific pattern inheritance is due to modifying loci rather than to the major loci alone. Even if one of these genes were homologous between species, evolution in *Heliconius* is so rapid that one would not expect to find homologies just by looking at the pattern

— molecular and linkage studies are also necessary. My own not-so-radical conclusion for *Heliconius* is that selection, rather than macromutation at homologous genes, has been primarily important for the evolution of mimicry.

Nevertheless, the book is both valuable and interesting. It is the first to collate a wide array of information about the biochemistry, development, morphology and genetics of lepidopteran patterns (there is no material on the molecular biology of development, because the relevant studies remain to be done). Some of the homology hypotheses in this book could be tested using cladistics: the inclusion of Don Harvey's radical reclassification of the Nymphalidae is an added bonus in this respect. Without the unifying hypothesis of variations around a ground-plan, perhaps the book would never have been written, and that would have been a great loss. Read this book, but keep alternative ideas in mind. □



Heliconius charitonias (zebra butterfly). The farthest butterfly is marked as part of a capture-release study. From *Sarapiquí Chronicle: A Naturalist in Costa Rica* by Allen M. Young. Smithsonian Institution Press, \$40 (hbk), \$16.95 (pbk).

underside elements. But this explanation ignores selection acting on the pattern's function on a partially translucent wing. The unmentioned alternative hypothesis is that the upperside band is homologous with only one or none of the aligned underside elements, just as alignments of nonhomologous elements are expressed within and between adjacent wing surfaces in *Kallima*. For *Heliconius*, Nijhout assumes that black, red and brown fields are "pattern" components of the ground-plan, whereas yellow and white are "background". As he points out, this might neatly explain the extraordinary genetic interaction elucidated by J. R. G. Turner in the forewing band of *H. melpomene*. But some *Heliconius* genes (in *H. erato* the Y gene; in *H. melpomene* the D and Yb genes) can replace red patches with yellow by 'overprinting'. Genes shaping overprinted patches affect both colours equally, and a colour switch here seems more likely than a mechanism obeying Nijhout's rules.

Nijhout ends with an evolutionary message. Pattern homologies suggest that mimicry and other colour patterns may evolve by macromutations at homologous genetic pattern elements. Sound

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Structure and functional properties of human general transcription factor IIE

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The general transcription factor IIE (TFIIE) is an essential component of the eukaryotic RNA polymerase II initiation complex. We have isolated human complementary DNA clones for both the subunits of TFIIE. Using purified recombinant proteins we find that both subunits are essential to form a stable preinitiation complex and to reconstitute basal-level and Sp1-activated transcription *in vitro*. Analysis of their predicted amino-acid sequences reveals several intriguing structural motifs that could provide insight into the role of TFIIE in transcription initiation.

TRANSCRIPTION initiation from eukaryotic protein-coding genes is a complex process requiring RNA polymerase II (PolII) and a cadre of other auxiliary transcription factors. These factors can be divided into two classes on the basis of their function: the general factors that are required for transcription of all PolII genes, and sequence-specific factors that are required for regulating expression. The general transcription factors and PolII form a specific multiprotein complex near the transcriptional start site by interacting with core promoter elements (reviewed in refs 1, 2). The most common core element is the TATA box, typically located 25–30 base pairs (bp) upstream of the transcriptional start site or initiator element. The presence of core elements alone seems to be sufficient for the formation of a productive complex that directs a low, unregulated (basal) amount of transcription. Sequence-specific transcription factors that bind to DNA-sequence elements located proximal and distal to the core promoter elements can dramatically enhance or repress transcription (reviewed in refs 3, 4).

The details of the biochemical pathway that leads to the synthesis of the first phosphodiester bond of the messenger RNA are unknown. To elucidate the events leading to initiation we have purified each of the components required to reconstitute the reaction *in vitro* and analysed its individual role in the process of transcription. *In vitro* reconstituted basal-level transcription from PolII gene promoters requires at least six distinct general factor activities, TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH, in addition to PolII, all of which have been extensively purified (O.F. *et al.*, unpublished data). Each of the basal factors seems to be essential to trigger accurate transcription initiation. Therefore, the complete characterization of the structure, function and interactions of the general factors with each other and the sequence-specific factors is seminal to unravelling the mechanisms that modulate transcription initiation.

Human TFIIE has been purified to apparent homogeneity and found to consist of two subunits of relative molecular mass 56,000 and 34,000 (M_r 56K and 34K)^{5,6}. The structure of TFIIE seems to be a heterotetramer ($\alpha_2\beta_2$), both subunits being required for optimal reconstituted basal-level transcription. But in the reconstituted transcription system used in those studies, only the purified 56K subunit seemed to have any TFIIE com-

plementing activity, with the 34K subunit having a stimulatory role. Here we report the molecular cloning and deduced primary structures of cDNAs encoding both the subunits, which reveal structural motifs that may provide clues to the function of these molecules and their role in transcription initiation.

Molecular cloning of TFIIE

Isolation of cDNA clones for both the subunits of TFIIE involved purification of the polypeptides to apparent homogeneity and obtaining a partial amino-acid sequence by microsequence analysis of proteolytic fragments. Oligonucleotide hybridization probes and polymerase chain reaction (PCR) primers against the peptide sequences were then used to isolate HeLa cell cDNA clones (see legend to Fig. 1). The 56K subunit sequence contains a single long open reading frame of 439 residues with a predicted M_r of 49.5K (Fig. 1a). RNA blot analysis identifies a single hybridizing poly(A)⁺ RNA species of about 3 kilobases (kb) (not shown). The 34K subunit sequence contains a single long open reading frame of 291 residues with a predicted M_r of 33K (Fig. 1b). RNA blot analysis reveals a single hybridizing poly(A)⁺ RNA species of ~1.5 kb, indicating that the 34K cDNA is nearly full-length (not shown).

Comparison of the predicted polypeptide sequences of both subunits with proteins in the database failed to identify sequences with a high global similarity to either subunit. But analysis did reveal several interesting and potentially important features (Fig. 2a–d, described below). The 56K subunit is acidic, having a predicted pI of 4.5, whereas the 34K subunit is basic, having a predicted pI of 9.5. In part, this results from the presence of stretches of acidic (56K) and basic (34K) residues located near the C termini of the molecules (Fig. 2a). Hyper-acidic stretches are a feature of several other nuclear and nucleolar proteins such as the high-mobility-group proteins and nucleolin. At the N terminus of the 56K polypeptide there is a sequence with the potential to form an amphipathic α helix.

Properties of recombinant TFIIE

To characterize the two polypeptides biochemically, they were independently over expressed in *Escherichia coli*. The purified recombinant subunits migrate at 34K and 56K, behaving indistinguishably from the native TFIIE subunits, but are slightly larger than their predicted M_r (Fig. 3a). Native TFIIE is thought to be a heterotetramer of 56K and 34K subunits on the basis of its apparent size determined by gel-filtration chromatography and the ratio of the subunits in the native complex^{5,6}. We therefore analysed the behaviour of the recombinant TFIIE (rTFIIE) subunits, either alone or together, by gel filtration chromatography (Fig. 3c). When the recombinant proteins were mixed and subjected to gel filtration, they behaved as a complex of about 230K, which is similar to the purified native TFIIE. On its own, the recombinant 34K subunit chromatographs as a dimer with an apparent M_r of 70K. By contrast, the 56K subunit behaves somewhat heterogeneously, ranging in size from 100 to 180K, with a peak of about 150K. Thus it seems to form a higher order complex consistent with the size of a dimer, but may also form nonspecific aggregates. To ascertain the ratio of the 56K and 34K subunits in the recombinant complex, known amounts

stable complex with the other general transcription factors on the AdMLP template.

Transcription

Previous studies showed that the purified HeLa cell 56K subunit of TFIIE alone has some TFIIE activity, whereas the 34K subunit is stimulatory^{5,6}. The ability of the recombinant subunits to substitute functionally for the endogenous HeLa cell TFIIE was therefore tested in a reconstituted transcription reaction using human TFIIA, TFIIB, TBP, TFIIF, TFIH and PolII. Reconstituted basal transcription from the AdMLP is dependent on the addition of an endogenous HeLa cell TFIIE to the reaction (Fig. 5a, lanes 1 and 2). Addition of either recombinant subunit alone fails to reconstitute transcriptional activity (lane 3–10). But when the recombinant subunits are added together, they promote levels of basal transcription comparable to the endogenous HeLa cell TFIIE (lanes 11–14). When the purified endogenous HeLa cell 56K subunit was assayed in the highly purified reconstituted system used here, it did not reconstitute transcription (not shown). Thus, in contrast to previous studies with purified endogenous TFIIE, we find that both subunits of TFIIE are required to reconstitute basal-level transcription.

We also tested the ability of rTFIIE to support activated transcription by site-specific activators such as Sp1. These experiments were especially important in light of the fact that recombinant human TBP cannot functionally substitute for the HeLa cell TFIID fraction to support Sp1-activated transcription in a reconstituted reaction, although the recombinant protein is fully competent at supporting basal-level transcription⁷. We therefore assessed the ability of the rTFIIE subunits to substitute functionally for endogenous TFIIE to support Sp1-activated transcription (Fig. 5b). Reconstituted transcription from a template containing six GC boxes and an initiator (G₄I; ref. 8) is efficiently activated on addition of Sp1 (lanes 1 and 2). When

endogenous HeLa cell TFIIE is replaced with rTFIIE, a comparable amount of Sp1-activated transcription is observed (lanes 3 and 4). As expected, this Sp1 response was dependent on the addition of TFIIE (lanes 5 and 6) and the presence of Sp1 sites (data not shown). The ability of TFIIE to support activation by the proline-rich activator CTF (ref. 9), was also tested. rTFIIE supports a moderate CTF-activated response, equal to that observed with endogenous TFIIE (data not shown). Thus, the 56K and 34K subunits of TFIIE are not only required for basal-level reconstituted transcription, they are also sufficient to reconstitute Sp1-activated (and CTF-activated) transcription.

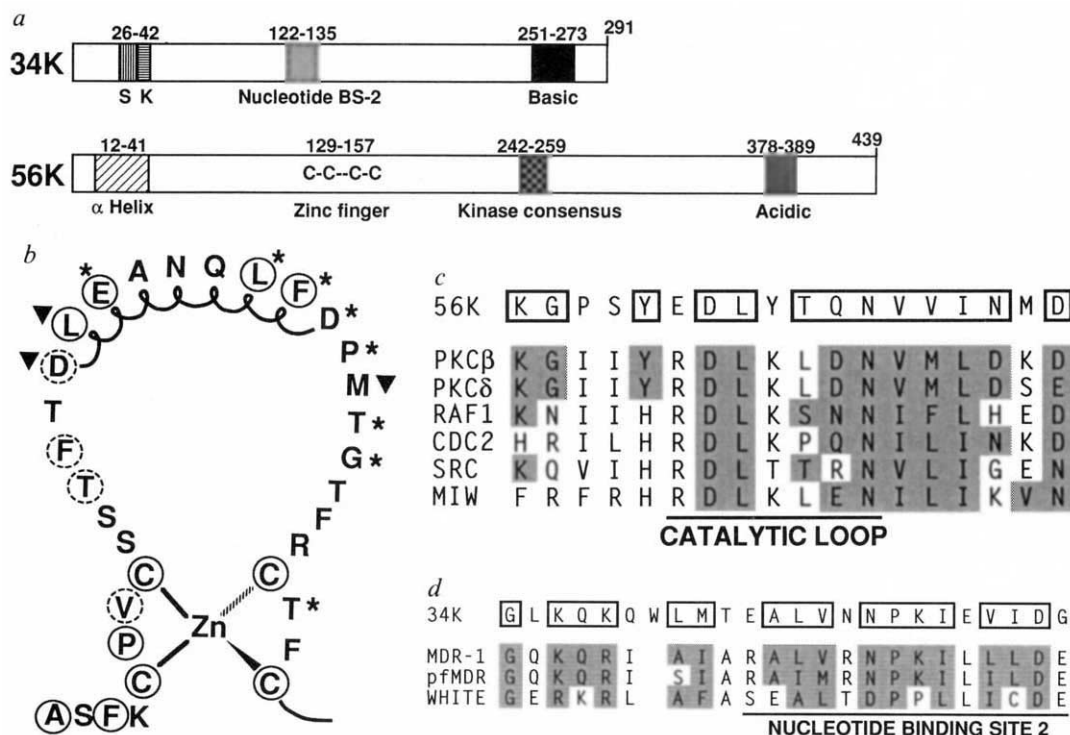
Discussion

E. coli-expressed recombinant proteins closely mimic the endogenous subunits in size on SDS-PAGE and gel filtration, in formation of a preinitiation complex, and in supporting reconstituted basal and Sp1-activated transcription. One apparent discrepancy between the properties of the endogenous and recombinant subunits was the inability of the recombinant 56K subunit alone to substitute functionally for TFIIE in a reconstituted transcription reaction. It was previously reported that the purified and renatured endogenous 56K subunit can support a low amount of reconstituted basal transcription, whereas the 34K subunit did not have any transcriptional activity on its own but could stimulate transcription. In the more purified assay used here, both subunits were essential for reconstituted transcription, and indeed when the purified endogenous 56K subunit was reassayed, it could not reconstitute transcription. This indicates that one or more of the transcription factor fractions used in the previous studies was contaminated with the 34K subunit of TFIIE.

A model of the formation of the preinitiation complex on a basal promoter is shown in Fig. 6. Gel filtration chromatography

FIG. 2 Structure and comparisons of the TFIIE subunits.

a, Schematic comparison of the 34K and 56K subunits. The residues spanned are shown above each feature: S, run of serine residues; K, run of lysine residues; nucleotide BS-2, similarity as shown in d; basic, region rich in basic residues; α helix, region predicted to form amphipathic α helix; zinc-finger, sequence that could potentially form a zinc-finger; kinase consensus, similarity as shown in c; acidic, region rich in acidic residues. b, Potential zinc-finger structure in the 56K subunit. Circles, residues identical (unbroken) or similar (broken) to the first zinc-finger of UvrA (ref. 12). Residues found to be identical (*) or similar (▼) to a region of the UvrB protein are indicated¹⁹. Curly line, region predicted to form an α helix. c, Comparison of the region of the 56K sequence containing a kinase consensus sequence²⁰ with segments of protein kinase C β and δ (PKC β and PKC δ ; Protein Identification Resource (PIR), access numbers B24664 and A28163), the kinase-related transforming proteins Raf1 and Src (PIR access numbers TVHUA and TVFFS), yeast cell division control protein 2 and mitosis inhibitor wee1 + (CDC2 and MIW, PIR access numbers TVP2 and A25962). Conserved residues are boxed and shaded. The region proposed to be the protein



kinase catalytic loop¹³ is underlined. d, Comparison of the region of the 34K subunit containing a similarity with nucleotide-binding site 2 of human *mdr*-1 protein (MDR-1), *Plasmodium falciparum* *mdr* protein (pMDR), and the *Drosophila white* gene product (WHITE; see ref. 21). Conserved residues are boxed and shaded.

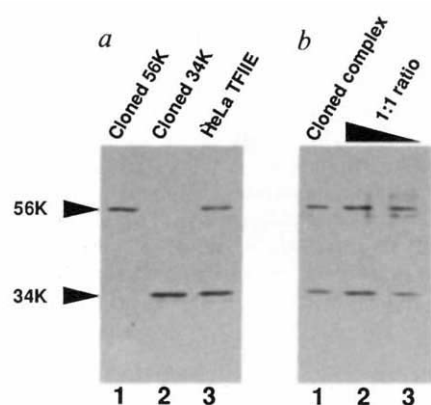
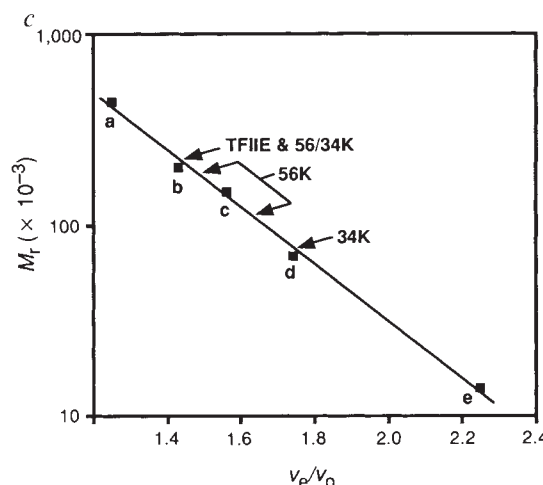


FIG. 3 Expression of cDNA clones in *E. coli* and subunit ratio in the cloned TFIIIE complex. *a*, The *E. coli*-expressed 56K and 34K subunits are similar in size to native HeLa cell TFIIIE. Lanes 1, 50 ng 56K subunit; 2, 50 ng 34K subunit; 3, about 100 ng native HeLa cell TFIIIE. *b*, The subunits of the cloned TFIIIE complex are in a 1:1 molar ratio. Lane 1, about 50 ng cloned TFIIIE complex obtained from a Superdex-200 column; lanes 2 and 3, 1:1 molar ratio of rTFIIIE subunits; lane 2, 50 ng 56K, 33 ng 34K; lane 3, 25 ng 56K, 16.5 ng 34K. *c*, Estimation of the native rTFIIIE M_r . The elution volume (V_e) over the void volume (V_0) of the endogenous HeLa cell TFIIIE (TFIIIE), the recombinant complex (56/34K), the 56K subunit alone, and the 34K subunit alone are indicated.

METHODS. TFIIIE recombinant proteins were expressed as described previously²². The recombinant 34K subunit was purified by collecting the 0.3 M KCl, 25 mM Tris-Cl pH 7.9, 0.1 mM EDTA, 20% glycerol, 10 mM β -mercaptoethanol, 0.1 mM PMSF (phenylmethylsulphonyl fluoride), 0.1 mM sodium metabisulphite (0.3 M buffer A, where 0.3 M refers to KCl concentration) flow-through from DEAE-Sepharose, diluting to 0.1 M buffer A then loading onto S-Sepharose, washing with 0.2 M buffer A and eluting with a 0.3 M



buffer A step. The 56K recombinant subunit was purified by chromatography on DEAE-sepharose in 0.2 M buffer A, then eluting with 0.3 M buffer A, applying to Q-Sepharose, then eluting with 0.5 M buffer A. The eluate was dialysed against 1.4 M ammonium sulphate in buffer A, specifically precipitating the 56K subunit. The concentration of each subunit was determined initially by the method of Bradford²³, these values being used for all experiments except the silver-stained gels shown in *a* and *b*, where amino-acid analysis of the most purified fractions was done. For analysis of the rTFIIIE complex, roughly equimolar amounts of purified 56K and 34K subunits were mixed and incubated on ice for 30 min, then analysed by gel filtration⁵. The native M_r of the recombinant proteins was estimated by interpolation from a curve constructed using five different protein standards: *a*, apoferritin, 440K; *b*, β -amylase, 200K; *c*, alcohol dehydrogenase, 150K; *d*, bovine serum albumin, 68K; *e*, cytochrome *c*, 14K.

suggests that the 34K subunit and possibly the 56K subunit may form dimers in solution. Moreover, both subunits seem to interact forming a heterotetramer ($\alpha_2\beta_2$) structure. Several motifs identified in the subunit sequences may form the basis of these presumptive dimerization and tetramerization domains. The 56K sequence contains a region near the N terminus having the potential to form an amphipathic α helix with hydrophobic residues at the 3 and 4 positions of the helix, consistent with the structure of a coiled coil. Interactions between the putative 56K and 34K dimers to form the heterotetramer molecule may occur through charge interactions as the subunits are acidic and basic, respectively, and in particular contain stretches of acidic and basic residues.

The DNA band-shift data presented here and previously indicate that TFIIIE associated with the transcription complex

after PolII and TFIIF (ref. 5). This observation and the previously described glycerol gradient analysis suggest that TFIIIE may interact directly with both PolII and TFIIF (ref. 10). General factor TFIIF also enters the preinitiation complex after PolII, TFIIF, and TFIIIE and it too may interact with the TFIIIE (O.F. *et al.*, unpublished data). Thus, the TFIIIE heterotetramer may be involved in protein-protein interactions with both PolII and TFIIF (Fig. 6).

DNaseI protection analysis of preinitiation complexes suggests that TFIIIE may protect the promoter region downstream of the transcription initiation site, although endogenous TFIIIE does not possess any known DNA-binding capability on its own¹¹. The 56K subunit of TFIIIE contains sequences similar to the zinc-finger motif found in a number of nucleic acid-binding proteins (Fig. 2*b*). Perhaps the putative zinc-finger of TFIIIE

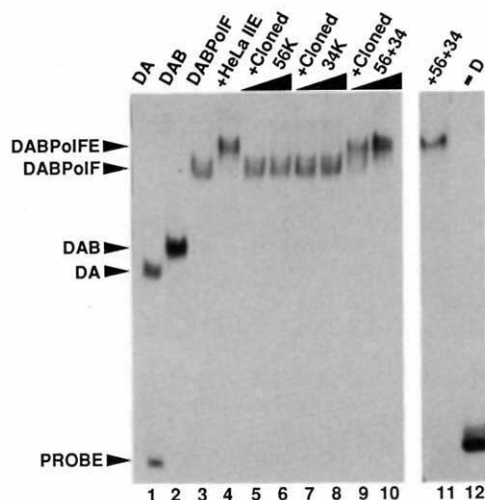
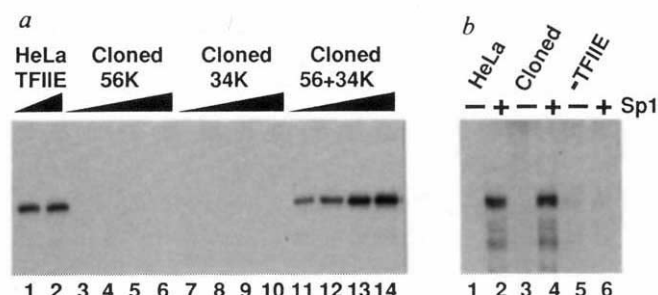


FIG. 4 Both subunits of TFIIIE are required to associate with the DABPolF DNA-protein complex. The protein components are as indicated at the top of each lane and the complexes formed are as indicated on the left. Lane 1, recombinant yeast TFIID and HeLa cell TFIIF; 2, as in 1 plus recombinant human TFIIF; 3, as in 2 plus HeLa cell RNA PolII and TFIIF; 4, as in 3 plus endogenous HeLa cell TFIIIE (0.6 pmol); 5 and 6, as in 3 plus either 10 (5) or 20 (6) pmol recombinant 56K subunit; 7 and 8, as in 3 plus either 10 (7) or 20 (8) pmol recombinant 34K subunit; lane 9 and 10, as in 3 plus either 10 (9) or 20 (10) pmol both the 56 and 34K subunits, lane 11 is a repeat of lane 10; 12, as in 11 without recombinant yeast TFIID, shows that all complexes are dependent on the presence of TFIID.

METHODS. The protein components, described in the legend to Fig. 6, were incubated with a fragment of the adenovirus major late promoter spanning the -40 to +13 region, using conditions described previously⁵.

FIG. 5 Both subunits of TFIIIE are required to reconstitute specific transcription *in vitro*. *a*, TFIIIE proteins as shown at the top of each lane were added to a specific basal level transcription complementation assay for TFIIIE activity on the AdMLP. Lanes 1 and 2, 0.3 (1) or 0.6 (2) pmol endogenous HeLa cell TFIIIE; 3–6, 0.075 (3), 0.15 (4), 0.3 (5) or 3.0 (6) pmol recombinant 56K subunit; 7–10, 0.075 (7), 0.15 (8), 0.3 (9) or 3.0 (10) pmol recombinant 34K subunit; lanes 11–14, 0.075 (11), 0.15 (12), 0.3 (13) or 3.0 (14) pmol both the recombinant 56K and 34K subunits of TFIIIE. *b*, Recombinant TFIIIE supports Sp1-activated transcription on a promoter containing GC boxes (G_6 ; ref. 8). TFIIIE proteins as shown at the top of each lane were added to a specific transcription complementation assay for TFIIIE in the presence or absence of Sp1. Lanes 1, 0.3 pmol endogenous TFIIIE; 2, lane 1 plus Sp1; 3, 0.3 pmol recombinant 56K and 34K subunit; 4, as in 3 plus Sp1; 5, no added TFIIIE; 6, as in 5 plus Sp1.

METHODS. Conditions for *in vitro* reconstituted transcription reactions were as described previously⁵. The transcription factors added to the reactions were partially purified HeLa cell TFIIA (DEAE 5PW fraction²⁴, 0.3 μ g), purified recombinant human TFIIIB (ref. 25) (0.03 μ g), purified recombinant human



TBP²⁴ (0.1 μ g), TFIIIE, either purified endogenous HeLa⁵ cell or purified recombinant human (as indicated), purified HeLa cell TFIIIF (ref. 26) (0.05 μ g), partially purified HeLa cell TFIIH (MonoS, 0.1 μ g; O.F. and D.R., unpublished results), and purified recombinant Sp1 (ref. 8) (0.2 μ g, as indicated).

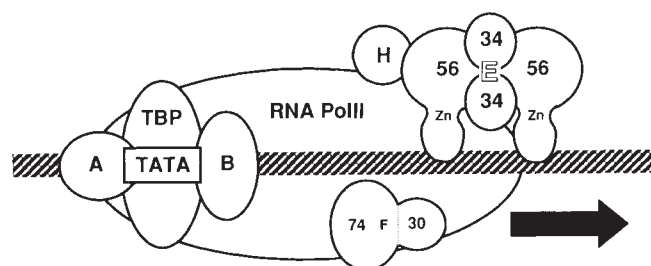


FIG. 6 Model of the preinitiation complex based on that of Buratowski *et al.*¹¹ The first critical step in the nucleation of the preinitiation complex is thought to be the binding of the TFIID complex to the TATA box element, through one of its subunits, the TATA-box-binding protein (TBP). The somewhat unstable binding of the TBP-TFIID complex to the TATA box may be stabilized by the binding of TFIIA (A). TFIIIB (B) is thought to bind next. RNA Pol II in the presence of TFIIIF (F) can then recognize and bind to this DNA-protein complex. TFIIIE (E) can then enter the preinitiation complex. This order of addition suggests that TFIIIE may interact directly with Pol II or TFIIIF, and indeed, a Pol II-TFIIIE complex has been observed by glycerol gradient analysis¹⁰. TFIIH (H) seems to enter the preinitiation complex after Pol II, TFIIIF and TFIIIE, and may also interact with TFIIIE (O.F. and D.R., manuscript in preparation). The putative zinc-fingers of TFIIIE are shown interacting with DNA (see text).

interacts with DNA only when positioned correctly in a preinitiation complex (as in Fig. 6). This region of the 56K subunit is very similar to the first zinc-finger of the *E. coli* UvrA protein, a component of the Uvr(A)BC excinuclease that binds preferentially to damaged DNA¹².

One domain of the 56K subunit revealed similarity to a highly conserved sequence found in nearly all protein kinases (Fig. 2c). When the sequence of TFIIIE surrounding this kinase domain consensus was compared with the protein databases, similarities with several protein kinase C molecules were identified. The similarity of the 56K subunit with these kinases extends beyond the previously identified conserved kinase domain sequence and indeed, in this region, the 56K subunit is more closely related to members of the protein kinase C family than this family is to most other kinases in the protein database. Recent crystal structure data suggests that this conserved region represents the catalytic loop of the kinase domain and the invariant Asp residue may be the catalytic base¹³. But the 56K sequence lacks several highly conserved sequences found in the catalytic domain of all protein kinases involved in nucleotide binding. The 34K sequence has a motif similar to nucleotide-binding site 2 of the multidrug-resistance protein¹⁴ (encoded by the *mdr-1* gene; Fig. 2d). From this sequence alignment, it

seems that the 34K and *mdr-1* proteins are as similar to each other in this region as the *mdr* proteins are to the *white* gene product.

What role in transcription initiation is suggested by these similarities with protein kinases and ATP-binding proteins? Previous studies have demonstrated a requirement for hydrolysable ATP or dATP for the conversion of the preinitiation complex to an active initiation complex, and a similar requirement for the phosphorylation of the C-terminal domain (CTD) of Pol II. Crude TFIIIE fractions have DNA-dependent ATPase activity¹⁰, although no such activity is associated with highly purified endogenous TFIIIE (ref. 5). The CTD kinase activity is unusual in that it may purify with and be highly dependent on the formation of the preinitiation complex, and phosphorylation of the CTD may be concomitant with the initiation of transcription¹⁵. Thus, this kinase activity is unusual in that its activity may be tightly controlled and dependent on both DNA-protein and protein-protein interactions. The availability of large quantities of purified recombinant TFIIIE will allow us to study the role, if any, of TFIIIE in the phosphorylation of the CTD and other components of the preinitiation complex and also the putative protein-protein and protein-DNA interactions of TFIIIE. □

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Large-scale superluminal motion in the quasar 3C273

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THE quasar 3C273, one of the first discovered¹, is optically the brightest example of a source with a one-sided jet. It was also the first object to display apparent superluminal motion on parsec scales², a phenomenon attributed to relativistic effects on the appearance of a jet moving close to the line of sight^{3,4}. The same explanation allows an intrinsically similar 'counter-jet', moving at high speed in the opposite direction, to be dimmed to invisibility. We have made observations at 1.7 GHz, using very-long-baseline interferometry with a global network of 16 radiotelescopes, resulting in a high-dynamic-range map of the jet with a ratio of peak brightness to r.m.s. noise level of 16,000:1. We fail to see a counter-jet, a result which is just barely consistent with the standard model of a superluminal jet. The jet extends out to 220 pc, and some models^{5,6} require that the relativistic bulk flow should continue along its entire length. Comparison with an earlier image shows that superluminal motion extends out to at least 120 pc, three times farther than previously noted⁷. Because different components emerge with different velocities^{7,8}, a third epoch of observations is needed to determine if any deceleration has occurred.

The quasar 3C273 is unusual in many respects, both in its very high optical and X-ray brightness, and also in its superluminal and other radio properties. On kiloparsec scales, it is unusual in that all the radio emission comes from a core coincident with the optical quasar with a jet and lobe on one side only⁹⁻¹¹. The object has been mapped with MERLIN¹⁰ at 1-arcsec resolution and shows a continuous 22-arcsec jet. Very-long-baseline interferometry (VLBI) maps at high frequency have shown the presence of compact knots of emission with apparent superluminal motion with respect to the core of the quasar^{2,7,8}.

It is generally agreed that the double extended lobes of extragalactic radio sources are powered by jets¹², which are themselves produced by the release of gravitational energy from a central black hole. In most powerful sources these jets, which extend to kiloparsec scales, are seen only on one side. It is still a matter of argument as to whether these kiloparsec jets are intrinsically one-sided or whether the backward jet is rendered invisible by bulk relativistic flow over these large scales¹³. In 3C273 the VLBI parsec jet displays apparent superluminal motion, which is taken as evidence for relativistic bulk flow close to the line of sight^{3,4}. It is thus of extreme interest to the understanding of extragalactic radio sources to examine how far this flow continues. The aim of our observations was to extend measurements on the VLBI jet from tens of parsecs to hundreds of parsecs, which requires maps of the highest dynamic range to detect regions of lower surface brightness in the jet. We hoped to achieve this by using the world array of radio-telescopes listed in Fig. 1 at 1.7 GHz rather than the more usual higher frequencies. At this frequency two effects help in the detection of the jet: first, the telescopes themselves have higher sensitivity; second, the jet is brighter with respect to the core, whereas at higher frequencies the core dominates. In this paper we adopt $H_0 = 100 \text{ km s}^{-1} \text{ Mpc}^{-1}$, giving a linear scale of 1.85 pc mas^{-1} .

Two sets of observations have been completed: the first, epoch 1986.18, used data from 12 stations (five in Europe, seven in the United States) and the second, epoch 1988.88, used 16 stations (eight in Europe, six in the United States, plus Arecibo and Hartebeesthoek). At the second epoch the better coverage of the aperture plane enabled a higher-resolution image, Fig. 1,

to be formed. Data were recorded using the National Radio Astronomy Observatory Mk II system and were correlated on the Jet Propulsion Laboratory/California Institute of Technology Block II Processor. Fringe visibilities were fitted using the program CALIB in the AIPS package, which calculates residual delay and residual fringe rate¹⁴. The calibrated data have been mapped¹⁵ using the procedure MAP in the Jodrell Bank system (R. G. Noble, A. Bailey and T.W.B., unpublished work).

The VLBI image in Fig. 1 has been made with the full resolution of the 16-telescope array. It has a dynamic range defined as the peak brightness to r.m.s. noise level away from the map centre of 16,000:1. This is the highest dynamic range VLBI map made to date, and provides an opportunity to study the material on 100 pc scales. The r.m.s. noise level of $600 \mu\text{Jy beam}^{-1}$ is close to the thermal noise level. The image enables us to put a limit on the jet/counter-jet ratio of at least 5,300:1 on any VLBI counter jet. The expected brightness ratio for two equal jets in opposite directions is $[(1 + \beta \cos \theta)/(1 - \beta \cos \theta)]^{2+\alpha}$, where β is the true velocity of the jet expressed as a fraction of the speed of light and θ is the angle to the line of sight. The observed spectral index α is 0.8 (ref. 8) giving a limit of $\beta \cos \theta \geq 0.91$ derived from this expression. Taking $\beta = \cos \theta$, which minimizes the true velocity for a given superluminal flow based on the ballistic model³, gives $\beta \geq 0.95$. Substituting these values into the apparent lateral velocity equation, $\beta_{\text{app}} = \beta \sin \theta / (1 - \beta \cos \theta)$ gives a value of 3.2, which is close to the observed superluminal motion. Thus the observed superluminal motion is just sufficient to hide an intrinsically equal-brightness counter-jet and the canonical model is not threatened³.

Figure 1 shows a discrete component at the core together with jet components at 7.5 mas identified with C3, 4, 5 of Unwin *et al.*⁸ and at 23 mas identified with C2. Although the jet is resolved across its width in regions farther from the core, most of the jet

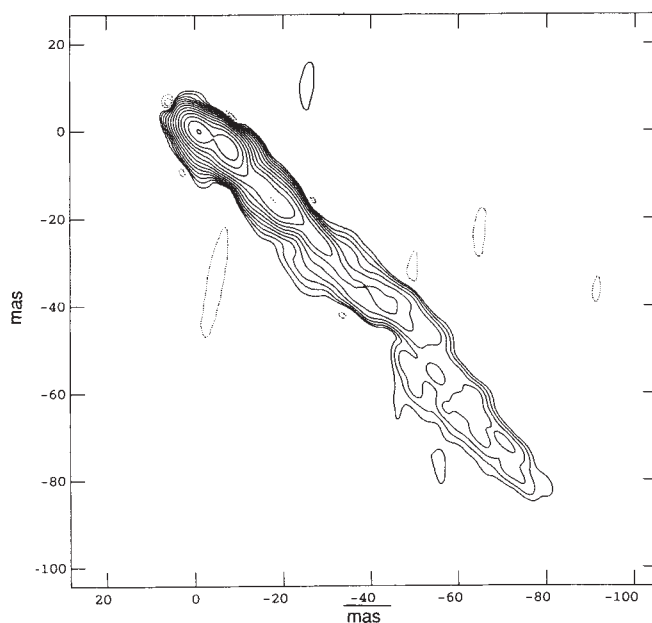


FIG. 1 VLBI map of 3C273 at 18-cm wavelength, epoch 1988.88, with 4.9×3.0 -mas resolution, position angle 31° . Peak brightness is $10.6 \text{ Jy beam}^{-1}$. No emission was seen outside the plotted area down to 2 mJy beam^{-1} . Contours are at equal logarithmic intervals of a factor 2 above a bottom contour of $2.5 \text{ mJy beam}^{-1}$. Negative (dashed) contours are at $-2.5 \text{ mJy beam}^{-1}$. The component at the origin of the map is identified as the core. No evidence for any counter-jet is seen. The radio emission displays 'wiggles' along its length and also is unresolved along its northern edge. The following stations took part in the observations: Jodrell Bank, Defford, Cambridge, UK; Onsala, Sweden; Crimea, Soviet Union; Medicina, Italy; Westerbork, Netherlands; Effelsberg, Germany; Hartebeesthoek, South Africa; Arecibo, Puerto Rico; Greenbank, North Liberty, Pie Town, VLA, Owens Valley, Haystack, United States.

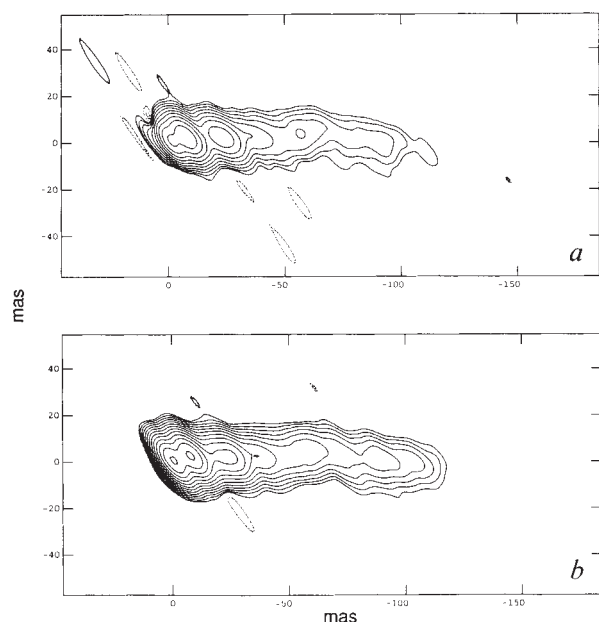


FIG. 2 VLBI maps of 3C273 at 18-cm wavelength (a) epoch 1986.18 and (b) 1988.88. The maps have been rotated by 46° . The resolution is 12.0×4.0 mas, position angle -10° with respect to North. For a and b, respectively, the peak brightnesses are 9.8 and $11.4 \text{ Jy beam}^{-1}$ and the contours are at equal logarithmic intervals of a factor of 2 above bottom contours of 10 and $2.5 \text{ mJy beam}^{-1}$. Negative (dashed) contours are at -10 and $-2.5 \text{ mJy beam}^{-1}$.

appears to be unresolved along its northern edge. This same phenomenon was seen in the kiloparsec jet, and an attempt was made to explain this on the basis of a wind¹⁰. The problem is more severe in the parsec jet because an even stronger wind would be required. The jet contains obvious wiggles, which are also seen in the kiloparsec jet¹⁰ although with wavelengths $\sim 1,000$ times longer. It is difficult to find a common mechanism for these wiggles over such a large change of scale.

The first epoch image has a lower resolution than that in Fig. 1 but only in the north-south direction, and thus for comparison, Fig. 2 displays both images with this lower resolution. The 7.5- and 23-mas component velocities (see Table 1) were measured using component fitting routines in AIPS. The errors in these velocities are not caused by noise but by mapping errors which are typically $\sim 2\%$ of the local brightness¹⁶. The rest of the jet from 40 to 120 mas is difficult to process in this way. Following the method of Walker *et al.*¹⁷, Fig. 3 shows a plot of differences in brightness between the two epochs along the jet plotted against gradients in brightness of the 1988.88 epoch. Shifts of the best-fit straight line from the origin are caused by any net change in brightness between the epochs. Scatter is caused by differential changes in brightness along the jet with change of epoch and mapping errors. The velocity is determined by shifts in the sharp edges and thus the best fit value of 0.85 mas was obtained by excluding points within 10 mJy beam^{-1} and $10 \text{ mJy beam}^{-1} \text{ mas}^{-1}$ of the origin. The error in Table 1 for this region is obtained directly from the scatter of the points from

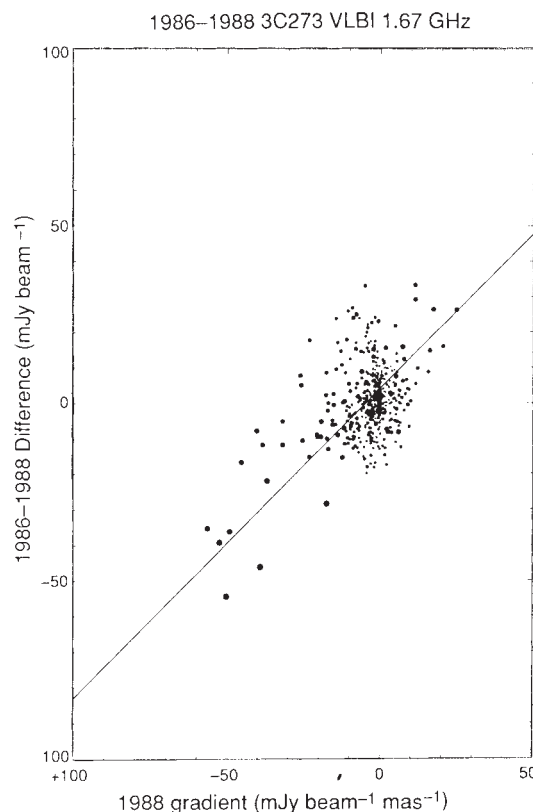


FIG. 3 A graph showing data of the weaker part of the jet from 40 to 120 mas from the core. The graph is constructed by plotting the difference for each pixel between the two epochs against the gradient in brightness along the apparent right ascension axis derived from adjacent pixels for the 1988 epoch. The plotted results have been averaged over adjacent pixels to account for oversampling of the beam. The symbol size is proportional to this averaged brightness. The gradient of 0.85 mas (solid line) is the best measurement of motion in this weaker part of the jet as described in the text.

the best-fit straight line and agrees well with the other errors in Table 1 estimated from map fidelity. The data are consistent with uniform flow in the region 40–120 mas, but the fit is strongly influenced by the brighter 40–65 mas part.

Components 5, 4, 3, 2 and the outer part of the jet have apparent velocities between $2.2c$ and $4.8c$ (Table 1). The apparent velocities, although a little less than those from higher-resolution, higher-frequency observations of the inner components, show that superluminal motion occurs throughout the length of the jet out to at least 120 pc. Because higher-resolution observations⁷ indicate that the core contains a contribution from emerging components, our lower apparent velocities may be due to our core being a blend of components. It is too early to say if we are seeing a deceleration as it is clear that components emerge at different velocities⁸. The outer 70–120 pc of the jet are, however, moving superluminally but marginally more slowly than the inner components. This result is important in the light of the continued debate on the speed of kiloparsec jets in extragalactic radio sources^{6,18,19}. In the galaxy 3C120, Muxlow and Wilkinson¹⁶ confirm apparent superluminal motion out to 75 pc, although they do not see the motion claimed by Walker *et al.*¹⁷ out to 2 kpc. We have shown that in 3C273 relativistic flow continues at least for the first hundred parsecs. Future observations with the resolution of Fig. 1 should enable the flow to be examined beyond 120 pc and will enable any deceleration of components to be measured. $\square$

TABLE 1 Component velocities

Component distance from core (mas)	Previous name	Apparent motion (mas yr^{-1})	Apparent velocity v/c (outward relative to core)
7.5	C3 + C4 + C5	0.52 ± 0.04	3.6 ± 0.3
23	C2	0.69 ± 0.04	4.8 ± 0.3
40–120	—	0.32 ± 0.05	2.2 ± 0.3

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A post-core-collapse model for the nucleus of M33

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RECENT high-resolution optical observations^{1,2} show that the nucleus of the nearby spiral galaxy M33 has a small core radius and an unusually low velocity dispersion. These parameters yield a central dynamical timescale of only a few tens of millions of years, suggesting that the nucleus of M33 has undergone core collapse, as is believed to occur in globular clusters. Here we propose a model for the post-collapse core of M33. By analogy with globular clusters, the formation of tight binary systems powers the re-expansion of the core, and we expect in particular that low-mass X-ray binaries form with a rate about equal to that of all galactic globular clusters combined. About a dozen such binaries should be present, and their combined emission may explain the large (10^{39} erg s⁻¹) and enigmatic unresolved X-ray emission from the nucleus of M33. In addition, tidal formation of cataclysmic binaries may lead to a nova rate of ≥ 1 per century. Numerous blue stragglers have probably formed through mergers caused by stellar collisions, but their density is probably too low to explain the blue colour of the nucleus. Young stars are the likely cause for the colour, and their presence may complicate the simple dynamical picture presented here.

The Local Group galaxies M31 and M33 contain a nucleus, that is, a small, high-density star cluster inside the core which acts as a separate dynamical component. Also, M32 contains a tiny core; although not a separate dynamical entity, it nevertheless has a central density and core radius similar to those in M31 and M33. These features would not be detectable if the galaxies were well outside the Local Group. Observed correlations between core parameters suggest that tiny cores should be common in low-luminosity galaxies^{3–5}, with^{6–10} or without¹¹ evidence for a central black hole. In the three well-studied cases of M31, M32 and our Galaxy, the central velocity dispersion is higher than that in globular cluster cores by about an order of magnitude. In M33, however, it is not. Recent observations^{1,2} give a central line-of-sight velocity dispersion of 22 km s⁻¹, only twice that in a typical globular cluster.

The observed limit^{1,2} on the core radius $r_c \leq 0.3$ pc. Using the virial theorem, or more specifically, the model of an isothermal sphere (equation (4-123) of ref. 12), the local density $\rho(0.3 \text{ pc}) = 2.0 \times 10^5 M_\odot \text{ pc}^{-3}$. The corresponding local two-body relaxation time (equation (8-71) of ref. 12, and equation (2.62) of ref. 13 with $m = M_\odot$ and $\ln \Lambda = 10$) is $t_{\text{rel}}(0.3 \text{ pc}) = 9.4 \times 10^7 \text{ yr}$. As 0.3 pc is an upper limit to the core radius and as the central

density is about three times that at the core radius, the central relaxation time is $t_{\text{rel}}(0) \leq 3 \times 10^7 \text{ yr}$ (see also ref. 2). Such a short relaxation time implies that the system probably has undergone core collapse.

We assume that the true core radius is $r_c \approx 0.1$ pc. There are three independent arguments for this value. First, simulations without primordial binaries show that cores oscillate after core collapse and that they spend most of their time near maximum expansion. They then typically contain 0.5–1% of the mass of a globular cluster. The corresponding core radius¹⁴ is $r_c \approx 0.1$ pc. Second, a significant population of primordial binaries will cause core collapse to halt and reverse at relatively large core radii^{15–17}. Again the value is $r_c \approx 0.1$ pc. Third, Hubble Space Telescope observations¹⁸ show that M15 has a core radius of $r_c \approx 0.1$ pc.

Table 1 summarizes our model of the core of the nucleus of M33. At these high densities, tidal capture is efficient^{19–20}, so we expect that several low-mass X-ray binaries (LMXBs) have formed. A quantitative measure of the formation rate is given by the collision number²¹; here $C = 10^9 = 0.26 C_{\text{tot}}$, where C_{tot} measures the total efficiency of tidal capture in all galactic globular clusters combined.

Tidal capture of neutron stars in the nucleus of M33 should, however, be much more efficient than in globular cluster cores, because the escape velocity is much higher in M33 than in globular clusters. Many neutron stars that would escape from a globular cluster will be retained in the nucleus of M33. There are two reasons for this. First, the total mass of the nucleus is larger than that of a typical globular cluster, and therefore its escape velocity is higher. Second, an escape from the nucleus of M33 would only be temporary: the radial orbits of escaping stars are likely to carry them back into the central parts of the nucleus often enough to drag them back in again through dynamical friction. Although these radial orbits will pick up some angular momentum, thereby increasing the pericentre to several core radii¹³, the change is too small to take the orbits altogether out of the nucleus. The dynamical friction cutoff is given by the finite age of the nucleus; this sets an upper limit on the radial extent of orbits that can be pulled back in by dynamical friction. If, say, half of all neutron stars produced have already drifted back into the core, then we have to multiply the collision number C by an extra efficiency factor $0.5/f$, where $f \approx 0.1$ – 0.15 is the fraction of neutron stars retained in globular clusters²¹. This could make the total efficiency of LMXB formation comparable to that of all galactic globular cluster cores combined.

The central X-ray source in the nucleus of M33 is puzzling^{22–25}, with a luminosity between those of X-ray binary stars and those of X-ray sources in low-luminosity active galactic nuclei. We suggest that the M33 nuclear X-ray source is a composite of a dozen or so LMXBs. The total luminosity of 10^{39} erg s⁻¹ is higher than the combined output of the ten galactic globular cluster X-ray sources, which is $\sim 10^{38}$ erg s⁻¹. But globular cluster X-ray sources in M31 are brighter than those in our Galaxy by nearly an order of magnitude (Verbunt *et al.*²⁶, and references therein), for reasons that are not understood. Therefore, as we do not know *a priori* whether X-ray sources in M33 should resemble those in M31 or our Galaxy, a combined luminosity of 10^{39} erg s⁻¹ is not implausible.

Our explanation for the central X-ray source is incompatible with considerable variations in its intensity; such variations were reported some time ago²³ but may be spurious^{25,27}. Although

TABLE 1 Model of core of M33 nucleus

r_c	0.1 pc
M_c	$2 \times 10^4 M_\odot$
σ	22 km s ⁻¹
ρ_c	$5 \times 10^6 M_\odot \text{ pc}^{-3}$

some LMXBs shine intermittently²⁸, the number of active sources at any time among ten or twenty LMXBs is not likely to fluctuate by more than a few. Therefore, we do not expect that the central X-ray source in M33 varies by more than a few tens per cent.

In addition to LMXBs, we expect that millisecond pulsars also will be formed more readily than in galactic globular clusters. More than twenty have so far been discovered in globular clusters. Searches are still far from complete. Therefore, an order-of-magnitude estimate for the population of such pulsars in the M33 nucleus is ~ 100 , which would make it even more extreme than the galactic globular 47 Tucanae, which contains at least 11 millisecond pulsars²⁹. Detections of individual pulsars in M33 are not feasible at present. The total radio emission from ~ 100 pulsars, which would be concentrated in the inner 0.1", may, however, be observable. If a central radio source were discovered in M33, the shape of its spectrum might indicate whether or not it is a collection of pulsars.

The high central density will lead to capture of white dwarf stars as well as of neutron stars. The enhancement with respect to globular clusters will be less marked for the former than for the latter, because white dwarfs do not generally receive a large velocity kick at birth, and are therefore likely to be retained even in globular clusters. But the high central density in M33 may cause cataclysmic binaries to form at a considerably higher rate than in even a highly concentrated globular cluster. Some of these will become recurrent novae. It is difficult to estimate the resulting total nova rate theoretically, but there have been a few novae seen in or near globular clusters over the past 100 years (see ref. 30 and references therein). The true nova rate in globulars may be higher. Therefore, a rough guess for the nova rate in the nucleus of M33 is ~ 1 event per century.

Finally, blue stragglers will form through mergers caused by physical collisions of stars in the dense centre of the nucleus, where interstellar distances are only ~ 0.005 pc ($\sim 1,000$ AU). As with cataclysmic variables, the enhancement compared with dense globular cluster cores will be modest. Even an optimistic estimate implies that $\leq 10\%$ of the stars in the core of the nucleus are merger remnants. As the total mass of the nucleus is at least an order of magnitude larger than the core mass, we do not expect blue stragglers to constitute more than $\sim 1\%$ of the nucleus. The total mass fraction of blue stragglers needed to explain the anomalous blue colour of the nucleus is $\sim 10\%$. Therefore, we join previous authors in rejecting the interesting possibility that the nucleus is composed of a globular-cluster-type old population. Some star formation must have occurred recently^{1,2,31-34}.

The presence of a small admixture of younger stars should not significantly modify our simple picture of the dynamical evolution of the nucleus. With such a small central relaxation time, all but the youngest stars will have reached local dynamical equilibrium. There is no indication that other complications, such as the presence of gas and dust in the nucleus, have an important role: the nearest H II regions are well outside the nucleus. Of course, past episodes of star formation may have changed the rate of dynamical evolution, possibly speeding core collapse. Also, young stars more massive than $1 M_{\odot}$ will slightly increase the dynamical effects of mass segregation. The situation is not, however, radically different from that of dense globular cluster cores, where physical collisions must have produced numerous merger products (possibly blue stragglers) as a result of physical collisions between stars. Thus, a relatively small fraction of young stars is unlikely to make a marked difference to our order-of-magnitude estimates.

We conclude that core collapse has probably taken place in the nucleus of M33. This may have created the anomalous central X-ray source by producing 10 or more low-mass X-ray binaries. In many ways, M33 may turn out to be an ideal laboratory for studying dynamical processes in star clusters: it is intermediate in properties between the densest galactic nuclei and the densest

globular cluster cores, and it is unusually inactive for a galactic nucleus. □

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Structural studies of membranes and surface layers up to 1,000 Å thick using X-ray standing waves

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THE X-ray standing wave (XSW) method, developed in the 1960s, was used originally to determine heavy atom positions in and on silicon and germanium single crystals¹⁻⁷. An X-ray standing wave generated by the interference of coherent incident and reflected beams excites X-ray fluorescence from the heavy atom, the intensity of which as a function of incident angle provides an indication of the atom's distance from the X-ray reflecting surface. The availability of X-ray mirrors and the ability to prepare layered synthetic microstructures has made possible the study of biologically relevant structures using the XSW technique on length scales of typically tens to hundreds of ångströms⁸⁻¹², allowing heavy atoms in such structures to be located with ångström or subångström resolution. Many model biological systems (such as Langmuir-Blodgett films, which mimic membranes) require access to still larger scales, but it is not obvious that an XSW will remain coherent

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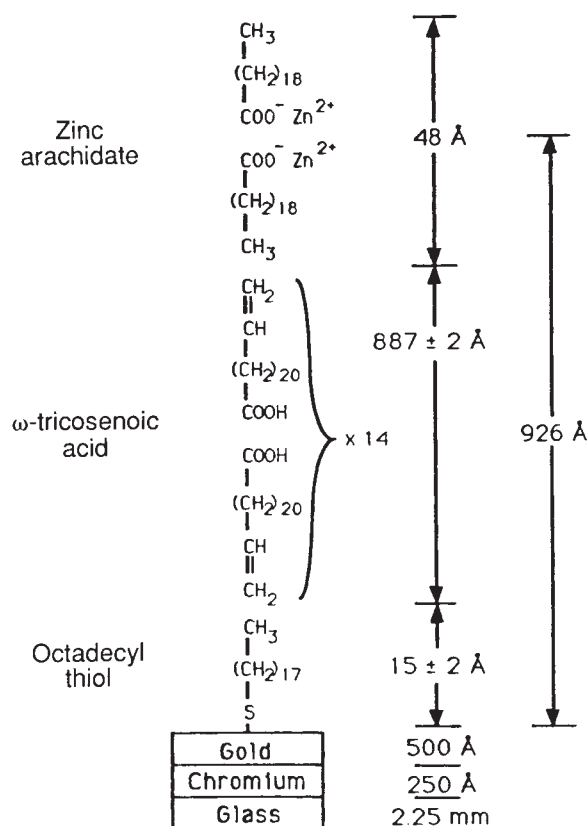


FIG. 1 Schematic of the gold mirror and deposited films. Gold was deposited as a 500-Å-thick film on a piece of chromium-coated float glass (5 cm × 2.5 cm × 0.2 cm) using thermoevaporation techniques and was plasma-cleaned before treatment with an octadecylthiol solution. The latter forms a stable, covalently bound monolayer with the hydrocarbon chains at the air-surface interface. Films of ω-tricosenoic acid and zinc arachidate were deposited by the Langmuir-Blodgett method. Transfer ratios were close to unity for each deposition. Film thickness was monitored after each deposition by ellipsometry. The dimensions shown were obtained from ellipsometry in the case of deposited organic films and from the manufacturer's specifications in the case of the mirror.

over such length scales. Here we report studies of a lipid multilayer system using the XSW method, in which we have been able to locate the metal atoms in a zinc arachidate bilayer with ångström resolution at a distance of almost 1,000 Å above the surface of a gold mirror. Our results indicate that the XSW technique should be useful for structural studies of supramolecular aggregates, receptor-ligand interactions and multi-membrane stacks, in which length scales of this order are encountered.

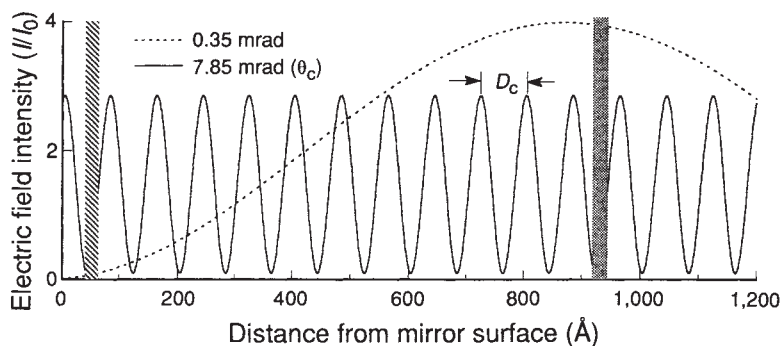
The strategy used to address the nature of the XSW electric field and its usefulness as a long-distance surface probe follows

(see Fig. 1). A hydrophobic gold mirror with 14 bilayers of ω-tricosenoic acid (ω-TA, sample A) and an inverted bilayer of zinc arachidate was prepared which posits the zinc layer between 900 Å and 1,000 Å above the mirror surface. By way of contrast, a second sample (sample B) was prepared that lacked the 14 ω-TA bilayers and had the zinc layer considerably closer to the gold mirror surface. From ellipsometry measurements, we estimated that the zinc layer was ~926 Å above the mirror surface for sample A and ~42 Å above the surface for sample B.

For this gold mirror system, the coherently related X-ray beams that generate the standing wave correspond to the incident X-ray beam and the beam totally reflected at the mirror surface below its critical angle, θ_c . Total external reflection occurs at the surface of the mirror when the incident angle, θ , is less than θ_c because the refractive index of X-rays is less than unity. We chose to use a gold mirror because the heavier the atoms constituting the mirror surface, the larger is θ_c , which in turn makes data acquisition easier and more accurate. The position of the nodes and antinodes of the standing wave relative to the mirror surface can be calculated for a given X-ray wavelength, λ , and θ , by Fresnel theory^{9,13}. XSW periodicity D is related to θ in the following way: $D = \lambda / (2 \sin \theta)$. Thus, at zero angle when the incident beam and the mirror surface are parallel, the XSW period is infinite in length, and the phase of the standing wave is such that a node coincides with the mirror surface. As θ is increased, the first antinode moves toward the mirror surface and is approximately coincident with the surface when θ reaches θ_c . At the same time the XSW periodicity decreases to $\lambda / (2 \sin \theta_c)$, which corresponds to the 'critical period', D_c . The other nodes and antinodes follow behind the first antinode like a collapsing bellows in the direction of the surface as θ is increased. For the example in Fig. 2, where a heavy-atom layer is situated *in vacuo* at ~925 Å above the gold mirror surface, it is apparent that a total of 12 complete XSW antinodes will have traversed the heavy atom layer as θ increases from 0 mrad to θ_c . The photoelectric effect, evidenced by X-ray fluorescence which is maximized whenever the XSW antinode coincides with the midplane of a heavy atom layer, can be used as a probe of the XSW electric field intensity normal to the surface as θ is varied. Conversely, the profile of the fluorescence yield can be used to calculate the position of the electric-field-sensitive heavy atom layer relative to the surface. It is more difficult to make XSW measurements on lighter elements such as oxygen, phosphorus and sulphur, because of their intrinsically weak fluorescence and poor detection efficiency at their characteristically low emission energies.

The results of the XSW measurements are presented in Fig. 3 for the two samples studied. Included in this figure are the experimental fluorescence yield (Fig. 3a and d) and reflectivity profiles (Fig. 3b and e) along with the calculated zinc distribution above the surface (Fig. 3c and f). The theoretical curves were determined by minimizing the χ^2 fit to the data in the region between 3 and 8 mrad. The goodness of fit demonstrates

FIG. 2 The height dependence of the normalized electric field intensity generated during specular reflection of a 9.8-keV (1.265-Å) X-ray plane wave from the surface of a gold mirror. Dashed and solid lines correspond, respectively, to the field profile with the mirror at an angle of 0.35 mrad (0.02°) and 7.85 mrad (0.449°, the critical angle for gold) with respect to the plane of the incident X-ray beam. At low angles, the first antinode of the standing wave is a considerable distance from the surface; at the critical angle, it is next to the surface. The positions of the zinc layers in samples A and B, at 925 Å and 50 Å, respectively, are indicated as shaded rectangles (see text for details). Calculations of electric field intensity, E , are based on Fresnel theory¹³ and assume the following: $E = 9.8$ keV; the mirror is *in vacuo* and the refractive index, n , of the gold film is the same as gold in bulk form ($n = 1 - \delta - i\beta$, where $\delta = 3.08 \times 10^{-5}$ and $\beta = 2.27 \times 10^{-6}$ at the photon energy).



that the XSW is well defined at $\sim 1,000 \text{ \AA}$ above the mirror surface and that it can be used to probe structure with dimensions on this same length scale.

The fluorescence yield profile from sample A (Fig. 3a) shows a single monotonically increasing fluorescence intensity from $\theta = 0$ mrad up to the critical angle of the film at $\theta = 2.2$ mrad. This is accounted for by the angle-dependent penetration of the evanescent wave¹⁴⁻¹⁶ through the upper organic-hydrocarbon layer in this region. From the calculations presented above for the idealized case of a zinc layer situated in a vacuum $925 \text{ \AA}$ above a gold mirror, we find that the first antinode of the XSW formed by the incident beam and that reflected from the gold mirror coincides with the zinc layer when $\theta = 2.3$ mrad. But because the intervening organic layer will, by refraction, bend the incident beam away from the surface normal, the angle of incidence at the gold mirror is now less than that of the incident beam striking the air-film interface and that calculated for the situation *in vacuo*. This effect shifts the fluorescence peaks (and

troughs) to higher nominal angles of incidence. The magnitude of this refractive effect lessens as the nominal incidence angle increases. At $\theta = 7.85$ mrad, however, the angle of incidence at the gold mirror surface below the organic layer is ~ 7.53 mrad. This corresponds to an XSW periodicity of $85 \text{ \AA}$ in the organic film, in contrast to $80 \text{ \AA}$ for $\theta = 7.85$ mrad in air. The net effect of the organic layer is thus to reduce the number of fluorescence peaks observed in the $0 < \theta < \theta_c$ region from 12 to $11\frac{1}{2}$. The theoretical curves shown in Fig. 3 take into account these refractive effects.

The decreasing fluorescence beyond the gold θ_c (Fig. 3a and d) is due to the steep drop in the reflected beam intensity in this region (Fig. 3b and e). The mismatch in amplitude between theoretical and experimental low-angle peaks in the fluorescence profile below 3 mrad (Fig. 3a) arises partly from a limited angular resolution in our measurements and partly from an extremely high calculated electric field in this region (see also ref. 17). Experiments are being conducted to test whether

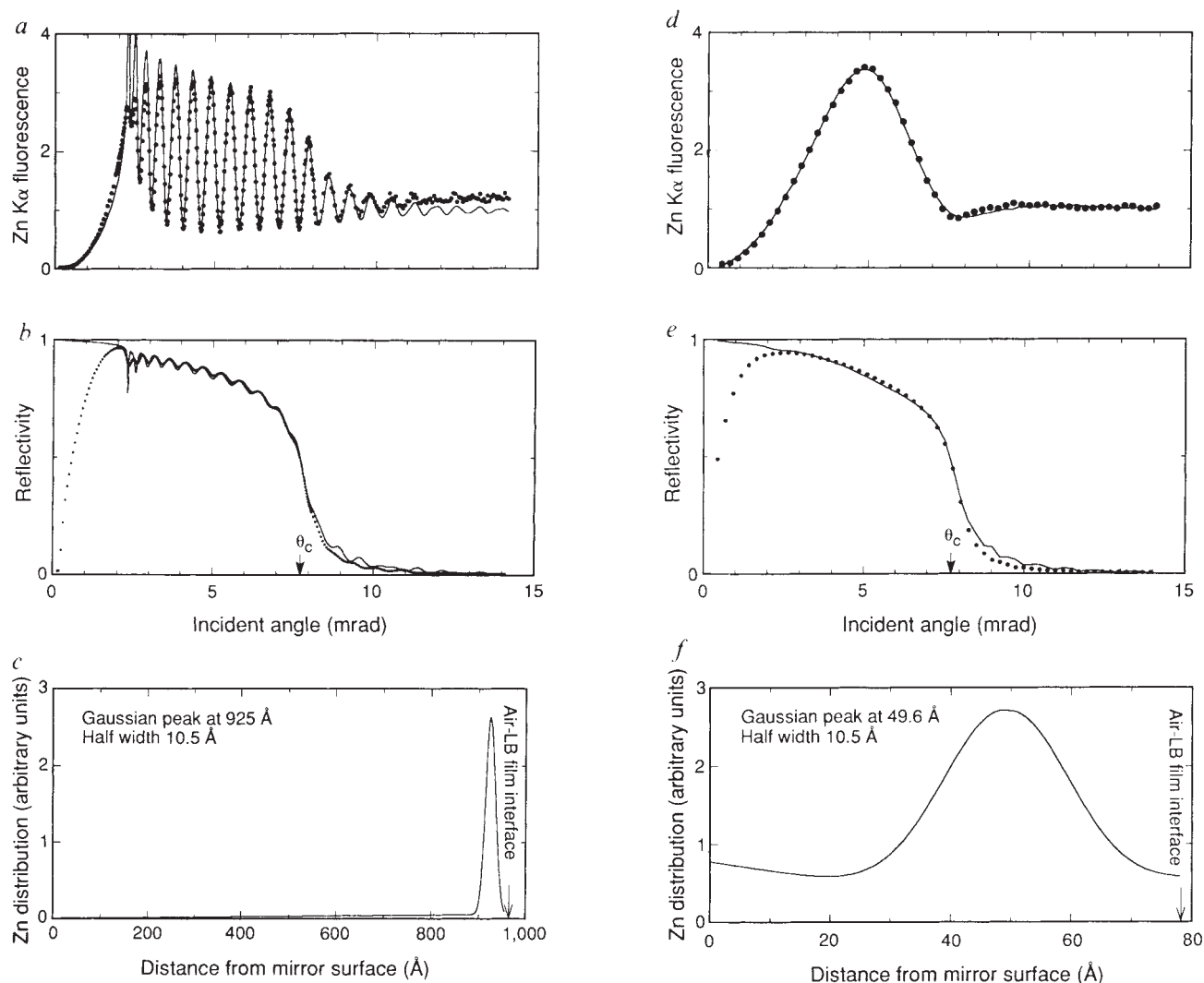


FIG. 3 The experimental (●) and theoretical (—) angular dependence at 9.8 keV of the zinc $K\alpha$ fluorescence yield (a, d) and the specular reflectivity (b, e) for samples with (a, b) and without (d, e) the 14 ω -tricosenoic bilayers. The reflectivity and angle were measured directly on an absolute scale. The fluorescence yields were normalized to the same area of illuminated film at all angles and scaled so that a fluorescence yield of unity is obtained beyond the critical angle. The calculated zinc distribution above the mirror surface is shown for samples with (c) and without (f) ω -tricosenoic acid. The total amount of zinc is normalized to unity. Fluorescence and reflectivity were measured with monochromatic light on the D-line at the Cornell High Energy Synchrotron Source. We used a germanium(111) monochromator to

tune the X-rays to 9.8 keV for optimal excitation of zinc $K\alpha$ fluorescence ($E = 8.9 \text{ keV}$). Fluorescence data were collected with a Si(Li) solid state detector with an energy resolution of $\sim 200 \text{ eV}$. For sample A, zinc $K\alpha$ fluorescence was obtained from a single-channel analyser; for sample B, we recorded complete fluorescence spectra at each incident angle using a multichannel analyser, and we measured the area beneath the zinc $K\alpha$ peak using a χ^2 -minimization gaussian fit and corrected for dead time. Above θ_c , scattering increases and shows up as background in the single-channel analyser data. The data for each point were collected in 3–15 s to ensure 1% counting statistics, with a beam $22 \mu\text{m}$ high and 2 mm wide, and the storage ring operating at 5.3 GeV and $35\text{--}50 \text{ mA}$ total positron current.

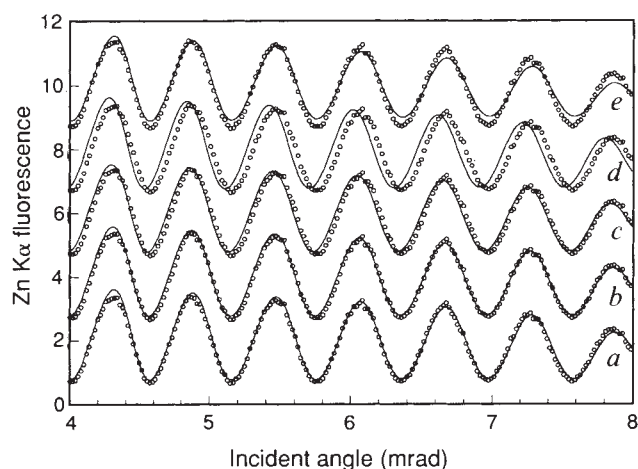


FIG. 4 The sensitivity of the fluorescence yield profile to the mean position of the heavy atom layer above the surface and to the distribution about the mean position is illustrated. The experimental data (●) for zinc K α fluorescence yield were taken from the central position (4–8 mrad) of Fig. 3a. Theoretical curves were generated by assuming a mean zinc position of 925 Å (a), 927 Å (b), 930 Å (c) and 935 Å (d) with a full width at half height of 21 Å. In (e) a mean zinc position of 925 Å and a full width at half height of 31 Å were assumed. Curves are displaced on the ordinate for clarity.

such large fields exist.

The modulation in the reflectivity curve between 2.3 and 7.5 mrad (Fig. 3b) arises from interference between reflections generated at the air-film interface and those generated at the film-mirror interface. An analysis of the reflectivity curve in this region provides an accurate measure of total film thickness (955 ± 5 Å). The reflectivity modulations continue out to higher angles, indeed beyond the point where data were collected in this study. The high-angle modulations contain information useful in reconstructing the electron density profile of the deposited film in the direction of the surface normal. We expect to concentrate on this reflectivity region in future work.

The agreement between experiment and theory is remarkably good as indicated in Fig. 3. This is illustrated more clearly in Fig. 4, which shows how changes in the mean position of the heavy atoms and spread about the mean position affect the goodness of fit. Sensitivity to mean position appears as mismatch in the phase of the modulation, whereas the distribution about the mean position affects the modulation amplitude. These data suggest that the XSW offers extremely good spatial resolution, of the order of several ångströms, on a length scale of just under 1,000 Å.

In conclusion, XSW generated by specular reflection from a gold mirror are well defined at $\sim 1,000$ Å above the mirror surface. The useful probing distance of the element-specific XSW is also of this length scale. At the same time, the spatial resolution of the XSW at such great distances from the surface remains in the range of several ångströms. Thus, variable-period XSW are likely to prove useful in surface structure studies, including membrane-membrane and receptor-ligand interactions, coatings, polymers, organic and inorganic thin films, and a variety of interfacial phenomena. With the advent of brighter X-ray sources and the use of wide-bandpass optics, kinetic studies on such systems will be possible. □

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The quasiquadrennial oscillation of Jupiter's equatorial stratosphere

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A NEW 11-year record of Jupiter's stratospheric temperature¹ shows an equatorial temperature oscillation with an apparent period of 4–5 years. Here we compare this oscillation to two long-period oscillations of zonal winds in the Earth's equatorial stratosphere, and propose that the same mechanism—forcing by the stresses of vertically propagating waves—is responsible for the oscillations on both planets. Jupiter's temperature oscillation has been observed for slightly more than two cycles and closely resembles the temperature signatures of the Earth's semiannual oscillation and quasibiennial oscillation. If the mechanisms responsible for these oscillations are indeed similar, Jupiter's oscillation indicates that there is significant vertical momentum transport due to equatorially trapped atmospheric waves, and it may provide a means for probing the poorly understood process of the generation of these waves by convection^{2,3}.

The temperature maxima and minima of the jovian oscillation are centred on the equator, with maxima in 1980, 1984 and 1989–90, and minima in 1982–83 and 1986–87. Figure 1 shows temperature profiles for two pairs of years having opposite phases of the equatorial temperature structure.

Zonal wind oscillations of the quasibiennial oscillation (QBO) have amplitude maxima near 25 mbar (~ 25 km), whereas those of the semiannual oscillation (SAO) are near 1 and 0.02 mbar (45 and 75 km)⁴. Both the QBO and SAO have instantaneous wind profiles in which eastward and westward zonal winds alternate in the vertical. Between zonal wind peaks of opposite sign are wind-shear layers. Time oscillations at a fixed height occur because this profile of alternating winds propagates downwards over time. On Earth, the wind-shear layers are uniquely related to temperature through the geostrophic and hydrostatic relations which together imply that equatorial temperature maxima coincide with positive (eastward) shear layers ($\partial u/\partial z > 0$, where u is eastward zonal wind speed and z is height) and equatorial temperature minima coincide with negative (westward) shear layers ($\partial u/\partial z < 0$) (ref. 5). Jupiter's rapid rotation rate, and the long timescale and zonal alignment of its wind and temperature structure, ensure that its equatorial mean zonal flow is also in geostrophic and hydrostatic balance. Consequently, its equatorial temperature maxima also coincide with positive shear layers, and equatorial temperature minima coincide with negative shear layers.

The Jupiter oscillation resembles the QBO in several respects: (1) long-period oscillation between equatorial shear zones of opposite sign, (2) strong equatorial confinement of the

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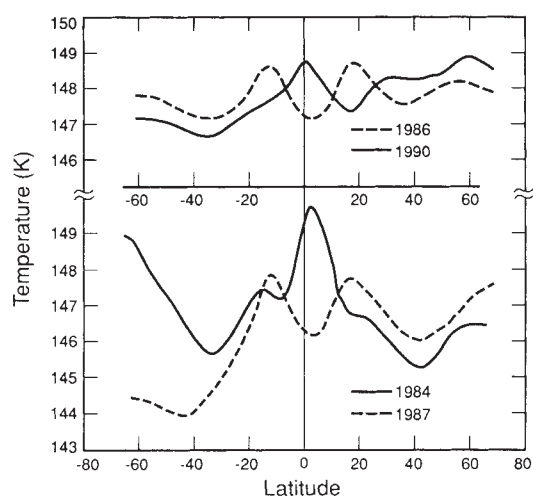


FIG. 1 Jupiter temperature data¹ for pairs of years with opposite phases of the equatorial temperature oscillation. Temperatures are attributed to layers centred near 20 mbar (upper) and 10 mbar (lower).

temperature anomalies and associated shear layers, (3) stronger eastward than westward shear layers and (4) a tendency for sharper equatorial confinement of eastward shear layers than westward shear layers⁶. In addition, the QBO and SAO are bounded on both sides of the Equator by temperature oscillations of opposite sign^{7,8}. As shown in Fig. 1, Jupiter's equatorial temperature oscillation is similarly bounded in latitude: temperatures near 15–20°N and S are relatively high when equatorial temperatures are relatively low, and *vice versa*. The observational similarities of Jupiter's equatorial temperature oscillation to the QBO suggest that it is produced by a similar mechanism. By analogy, we propose quasiquadrennial oscillation, or QQO, as a term for the Jupiter oscillation. Of course, additional years of temperature data may prove this to be a misnomer.

The equatorial waves responsible for the stresses that accelerate the zonal winds of the QBO and SAO are believed to be generated by convection in the Earth's tropical troposphere^{9,10}. Waves with eastward phase propagation carry eastward momentum upwards, and waves with westward phase propagation carry westward momentum upwards. The former propagate preferentially through westward zonal mean winds, the latter through eastward zonal mean winds. On Earth, dissipation by radiative damping or turbulence reduces the momentum flux as these waves travel upwards, especially where the difference between the zonal wind and wave phase speed decreases upwards and

approaches zero. In eastward shear zones, the resulting net eastward stress accelerates the zonal flow toward the east, and net westward stress accelerates the flow toward the west in westward shear zones. As a result, the entire pattern, including shear layers and alternating eastward and westward wind maxima, descends over time. Wave stresses may force descending shear zones in the same way on Jupiter, except that radiative damping is too weak to be important.

Planetary-scale Kelvin waves contribute to the eastward stresses of both the SAO and the QBO, whereas Rossby-gravity waves contribute to the westward stresses of the QBO¹¹. In addition, internal gravity waves contribute to both westward and eastward driving for both oscillations¹². In a recent modelling study of the QBO, the required amount of wave driving could be reconciled with observed Kelvin and Rossby-gravity wave amplitudes only if the latter were supplemented by internal gravity waves (M. Takahashi and B. Boville, personal communication). Oscillating zonal flow driven by internal gravity-wave stresses has also been demonstrated in the laboratory¹³. The SAO is locked to the semiannual cycle and its westward phase is forced in part by the annually oscillating meridional circulation, which produces semiannually varying momentum forcing on the Equator¹⁴. The QBO, on the other hand, is not precisely phase-locked to any periodic forcing. The time between successive QBO maxima has varied from 22 to 34 months.

Whereas the westward wind phases of the QBO and SAO can be driven by more than one mechanism, only stresses due to vertically propagating waves are known to be effective in driving the eastward phase. This uniqueness, coupled with the strength of the eastward phase on Jupiter, is a strong indication that the Jupiter oscillation belongs to the same family of phenomena. Although the apparent period of the QQO is not far from half of Jupiter's 11.8-yr orbital period, the QQO is probably a closer analogue of the QBO than the SAO. This is because the annually forced meridional circulation, which has an important role in the SAO, is likely to be weak on Jupiter's equator. Estimates of zonal mean wind shear and shear-layer latitudinal width are given in Table 1.

The years 1984 and 1987 have the largest positive and negative equatorial temperature signatures of any of the years in the record. Temperatures were inferred from radiances in the 7.8- μ m methane band and correspond to a contribution function centred near 10–20 mbar. The exact pressure level where the methane band contribution function peaks in Jupiter's stratosphere is somewhat sensitive to the vertical temperature profile. By examining equatorial limb-darkening profiles in this band, we estimated that the peak of the contribution function was near 10 mbar in 1984 and 1987, and near 20 mbar in other years. On this basis, temperatures were assigned to the 10-mbar level for 1984 and 1987, and to the 20-mbar level for other years¹. The stronger signatures of the QQO in 1984 and 1987 may arise because shear amplitudes generally grow stronger with elevation

TABLE 1 Properties of the equatorial shear zone

Year	Shear (m s ⁻¹ per scale height)	Half-width to zero point of shear (° lat)	Latitudinal extent of fit to data (°)
1984, eastward	+32	12	10
1986, westward	-11	16	16
1987, westward	-14	14	16
1990, eastward	+15	12	16

To facilitate estimation of the wind shear on the equator, data from opposite sides of the equator were averaged to produce synthetic profiles symmetric about the equator. Fourth-order polynomials were then fitted to the data. The data were fitted out to the latitudes indicated, which were chosen to optimize the fit of points closest to the equator. The equatorial geostrophic vertical wind shear per scale height (23 km for this region of Jupiter's atmosphere) was evaluated from $H\partial u/\partial z = -[R/(2\Omega a)]\partial^2 T/\partial \phi^2$ where T and u are zonal mean temperature and eastward zonal wind, H is pressure scale height, R is the gas constant, Ω is the planetary rotation rate, ϕ is latitude, and a is the planetary radius.

TABLE 2 Estimated properties of equatorial wind oscillations

	Pressure (mbar)	Density (kg m ⁻³)	Amplitude (m s ⁻¹)	Width (° lat)	Stress (N m ⁻²)
QBO	50	0.087	18	20	7×10^{-4}
SAO	1	0.0014	15	35	10^{-5}
QQO	10	0.0017	23	13	4×10^{-5}

Data on the QBO are from ref. 11 and on the SAO from ref. 14. Assuming a wave driven QQO, a lower limit to the amplitude of the wave stress τ in the layer within a scale height of Jupiter's 10-mbar level was derived from the vertical momentum balance, $\tau \approx -\rho H \partial u / \partial t$, assuming that the momentum is deposited in a layer roughly one scale height deep (ρ is density). The estimate of QQO stress was obtained by multiplying the 1987–1984 difference by π and dividing by the time interval.

through the layer sampled, or alternatively, they may simply provide evidence of the strongest vertical shears occurring in the 11-year observing period.

Some comparisons of the wind oscillations are given in Table 2. For the QJO, the stress is estimated from the 1984 and 1987 data, but the amplitude and corresponding stress may still be low because the system may be more than one scale height deep, and the estimated curvature of temperature on the equator, from which the estimate of equatorial shear was derived, may be low because of the finite resolution of the measurements, $\sim 5^\circ$ latitude.

The average radiative flux emitted by Jupiter is $\sim 15 \text{ W m}^{-2}$. Because $\sim 40\%$ of the flux is internally generated, a large fraction of this must be carried upward by convection in the troposphere. In the Earth's tropics, the upward flux carried by convection is $\sim 50 \text{ W m}^{-2}$, on average, within a factor of 10 of that of Jupiter. Although we cannot claim precision for our estimates for either convective heat flux or wave stress, the rough comparability of the convective energy fluxes and stress estimates is consistent with the hypothesis that the equatorial oscillations on both planets are driven by convectively generated vertically propagating waves, provided that the efficiency for convective generation of waves is the same order of magnitude on both planets.

The stress is also responsible for driving circulation in the meridional plane. This circulation generates, by adiabatic vertical motions, the temperature variations required to maintain geostrophic and hydrostatic balance, and it is probably responsible for the temperature variations observed near $15\text{--}20^\circ \text{ N}$ and S (Fig. 1). To ensure mass continuity, rising motion and adiabatic cooling on the equator must be balanced by subsidence and adiabatic warming at higher latitudes, and *vice versa*. The width of the circulation must correspond roughly to the latitudes of the maxima of temperature variation. Because of Jupiter's rapid rotation rate, the latitudinal width of equatorial waves likely to be responsible for the forcing is probably substantially smaller than the observed width, $\pm 15\text{--}20^\circ$, of the meridional circulation, so the latter may be indicative of the strength rather than of the width of the forcing¹⁵.

Confirmation of the oscillation and refinement of its period and structure will require additional observations. Mapping the planet at higher spatial resolution would enable a more accurate determination of the latitudinal extent of each phase of the oscillation and of the strength of the associated vertical shear of zonal wind at the equator. Verification that the oscillation descends over time will require successful strategies for mapping temperature at different stratospheric levels. $\square$

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Sensitivity of the Earth's climate to height-dependent changes in the water vapour mixing ratio

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THE atmospheric water vapour feedback is thought to amplify the global climate response to increased concentrations of greenhouse gases¹. As the oceans and atmosphere warm, there is increased evaporation, and it is generally believed that the additional moisture then adds to the greenhouse effect by trapping more infrared radiation. Lindzen^{2–4} has suggested that climate models overestimate this response; he argues that increased convective activity in a warmer climate will lead to a drying rather than a moistening of the upper troposphere (but see refs 5–7 for other views). An important part of Lindzen's argument is that it is only changes in upper tropospheric water vapour that can alter the radiative budget of the atmosphere significantly, and hence contribute to the water vapour feedback. Here we use radiative transfer calculations to show that this seems to be true if the water vapour concentration is perturbed by a constant absolute amount at all heights. But observations of the seasonal change in water vapour concentrations^{6,8} suggest that the climate response to increased concentrations of greenhouse gases may be closer to a constant relative change at all heights, corresponding to much larger absolute changes in the lower troposphere. We find that the Earth's radiation budget is most sensitive to changes in lower tropospheric water vapour concentrations, when such relative perturbations are considered.

Using a radiative–convective climate model, Manabe and Wetherald⁸ showed that the sensitivity of their modelled climate

to perturbations in greenhouse gas concentration was increased considerably if the water vapour amount was allowed to change in such a way as to maintain constant relative humidity. They reasoned, using the observed seasonal cycle in support, that increased temperatures would lead to a greater concentration of water vapour in the atmosphere; because water vapour is itself a strong greenhouse gas, this increase leads to a further warming. They employed a simple analytical prescription of the vertical variation of relative humidity. Subsequently, general circulation models (GCMs), with a more explicit representation of the water vapour budget, have yielded similar results^{1,6,9}.

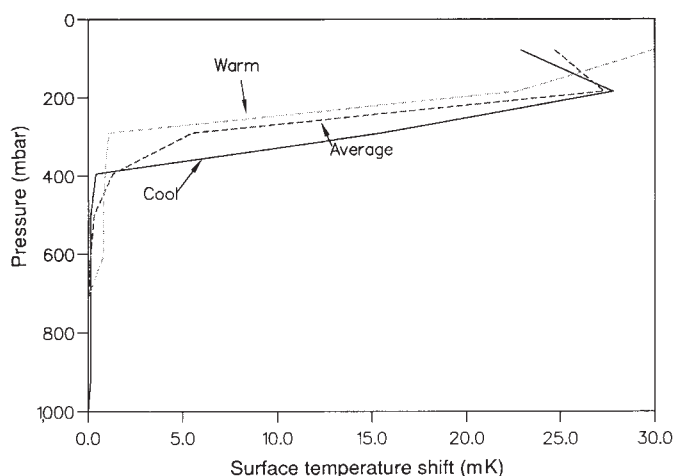


FIG. 1 Surface temperature changes (mK), obtained from a radiative–convective climate model, which result from an increase in water vapour mixing ratio by 0.001 g per kg in individual 50-mbar slabs centred at every 100 mbar level. The three atmospheres used are described in the text. The vertical axis shows the pressure at which water vapour is perturbed.

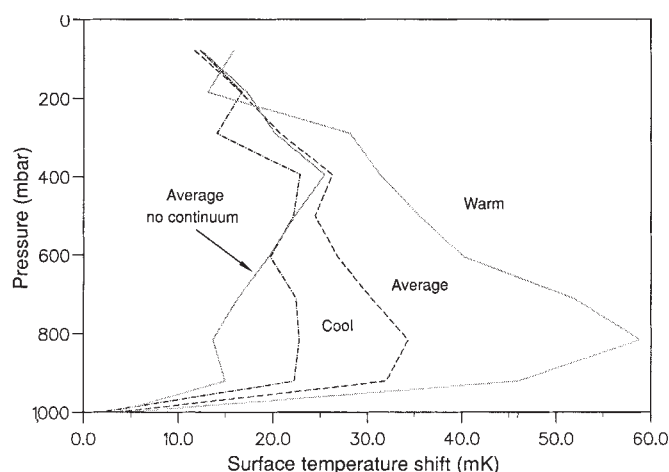


FIG. 2 As Fig. 1, but for an increase in water vapour mixing ratio of 10% in individual 50-mbar slabs. Results for the average profile with the continuum absorption removed are also shown.

Satellite and surface observations have yielded a strong relationship between sea-surface temperature and total atmospheric water vapour¹⁰ and the atmospheric greenhouse effect¹¹.

One weakness in modelling the water vapour budget, particularly in the upper troposphere in the tropics, is that much of the transport from below is achieved by convective-scale motions; in GCMs these are sub-grid-scale phenomena which must be parameterized in some way. Lindzen² has questioned the validity of the parameterizations currently in use and has suggested that water vapour in the upper troposphere will decrease should the strength of convection increase in a warmer world. Combining this with the claim that the climate is insensitive to changes in lower tropospheric water vapour, Lindzen suggests that the sign of the water vapour feedback should actually be negative.

We have explored the sensitivity of a simple climate model to perturbations in water vapour at various altitudes. We used a conventional radiative-convective model⁸ with a 6.5 K km^{-1} convective adjustment, a relatively simple three-band radiative transfer scheme¹² (based on refs 13 and 14) and a multi-band shortwave scheme¹⁵; the atmosphere was divided into twenty 50-mbar slabs, and global mean cloudiness⁸ was specified. From an equilibrium profile of water vapour derived using the Manabe and Wetherald⁸ expression, the water vapour amount was increased in individual 50-mbar slabs, first by the same absolute

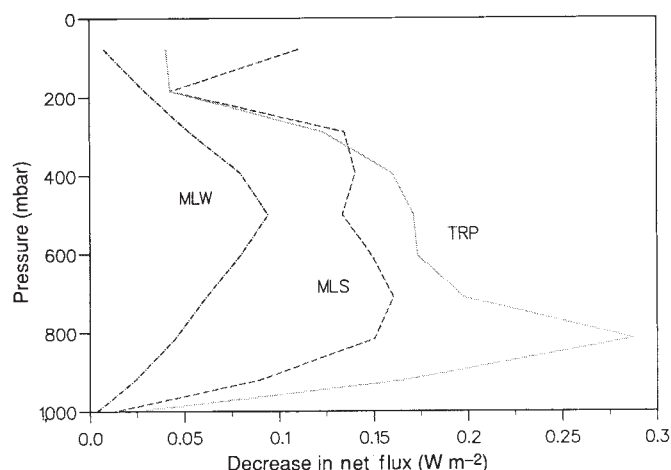
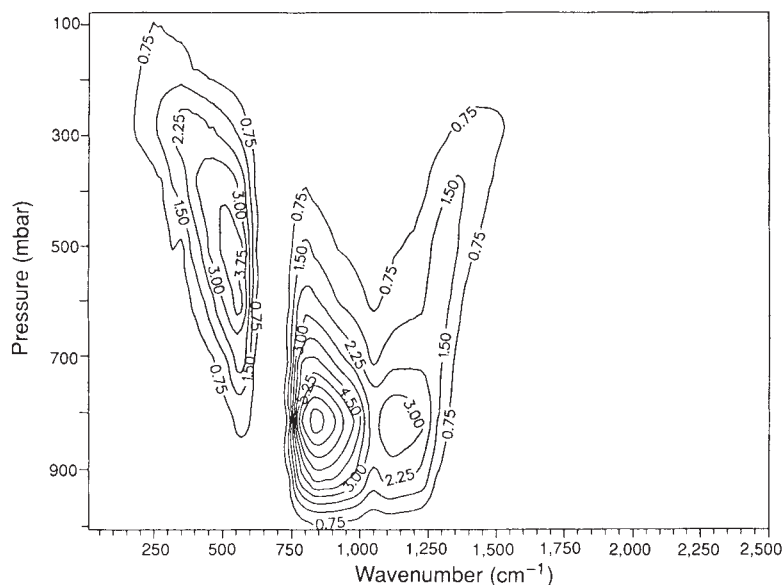


FIG. 3 Decrease in net thermal infrared flux (W m^{-2}) at the tropopause for clear sky conditions for an increase in water vapour mixing ratio of 10% in individual 50-mbar slabs, using a narrow-band model. Again, the vertical axis shows the pressure at which the water vapour is perturbed. Three standard atmospheres are used, mid-latitude winter (MLW), mid-latitude summer (MLS) and tropical (TRP).

amount of water vapour (0.001 g per kg) and then by the same relative amount (10%) and the new equilibrium temperature found; this process was repeated at every second 50-mbar slab (to reduce computer time). (A repeat of the calculations for decreases of the same amounts showed the model to be linear to within $\sim 10\%$). The sensitivity of the surface temperature to these perturbations was then examined. As the response might be expected to depend on the total amount of water vapour in the atmosphere, we present results for three profiles generated by altering the incident solar radiation. These are referred to as the cool, average and warm profiles and have surface temperatures of 277 K, 287 K and 298 K, respectively, and total water vapour columns of 0.83 cm, 1.76 cm and 3.71 cm respectively.

Figure 1 shows the sensitivity of the surface temperature to the perturbations of 0.001 g per kg in each alternate layer. The surface temperature seems insensitive to changes in water vapour anywhere at pressures higher than ~ 400 mbar (altitudes lower than ~ 6 km), and in general the moister warm profile with its higher infrared opacity is least sensitive. It might be concluded that any water vapour perturbations in the lower troposphere are unimportant to climate sensitivity, an inference that seems

FIG. 4 Change in net thermal infrared flux (mW m^{-2} per 10 cm^{-1}) for the tropical profile in Fig. 3, decomposed into the effect in each 10 cm^{-1} interval used in the narrow-band calculation.



to have been drawn by Lindzen².

But an entirely different picture is shown in Fig. 2, which illustrates the sensitivity of surface temperature to 10% increases in water vapour in every second layer. Because the water vapour mixing ratio changes by several orders of magnitude between the surface and upper troposphere, the absolute perturbation likewise changes by orders of magnitude. The model is now most sensitive to changes in water vapour at 800 mbar, and the moister warm profile is by far the most sensitive. In climate model simulations of increased carbon dioxide concentrations^{6,9}, the response is closer to a relative perturbation that is constant with height. Radiosonde⁸ and satellite⁶ observations of the seasonal variation of water vapour concentration also indicate a similar change. Thus calculations of the effects of relative changes are probably more applicable than those for constant absolute changes.

Earlier studies^{16,17} have emphasized the importance of the continuum absorption at 800–1,200 cm⁻¹ to climate sensitivity. In Fig. 2, we have added a fourth curve, representing the sensitivity of the average profile when this continuum absorption is removed. The response in the lower troposphere is greatly reduced but remains within a factor of two of the upper tropospheric peak. Because water vapour also absorbs in the near-infrared region of the solar spectrum, a further experiment was done in which the water vapour for the solar part of the calculation was held fixed, so that the relative importance of perturbations in the solar and thermal infrared regions of the spectrum could be explored. In this case (not shown), the climate sensitivity was reduced by typically 10%. We have also found that a simultaneous perturbation in different layers yields a result within ~10% of that obtained by summing the effect of individual perturbations.

The thermal infrared radiative transfer scheme used to generate the above results is rather simple and allows only a rather crude assessment of the importance of different spectral regions. Separate calculations have been performed with a far more detailed radiative transfer scheme. The model¹⁸ resolves the thermal infrared spectrum between 0 and 2,500 cm⁻¹ into 10-cm⁻¹ intervals. It was computationally impractical for us to do the radiative-convective calculations with such a model. Instead, using standard atmospheres¹⁹ and clear skies, we perturbed the water vapour at each level and calculated the change in net flux at the tropopause; the normal definition of climate forcing^{1,20} allows for the stratosphere to adjust to the imposed forcing, but we found that this had a negligible impact on our calculated flux changes. The effect of 10% relative perturbations on mid-latitude winter, mid-latitude summer and tropical atmospheres is shown in Fig. 3. The tropical, and most moist, profile is again generally the most sensitive to relative perturbations, peaking at 800 mbar. Finally, Fig. 4 shows the contribution of each 10-cm⁻¹ spectral interval to the total net flux change for the tropical profile. This plot shows that at the lowest levels it is the continuum absorption between 800 and 1,200 cm⁻¹ that is the dominant contributor and responsible for the peak at 800 mbar. As the water vapour mixing ratio decreases with height, the contribution from the continuum diminishes. The rotational bands of water vapour at wavenumbers less than 650 cm⁻¹ become most important, with a lesser contribution from the vibration-rotation bands at wavenumbers greater than 1,200 cm⁻¹. The region between ~650 and 750 cm⁻¹ is rendered unimportant by the strong carbon dioxide bands.

Contrary to the suggestions of Lindzen²⁻⁴, we have shown that perturbations of water vapour in the lower troposphere are indeed important for climate sensitivity. Any assessment of the effect of water vapour feedback must take into account changes at all levels in the troposphere; even if the notion of upper tropospheric drying came to be accepted, increases in lower tropospheric water vapour amount could still render the overall water-vapour feedback positive. □

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Neodymium isotope evidence for ultra-depleted mantle in the early Proterozoic

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THE episodic extraction of juvenile continental crust from the Earth's mantle over the past 4 Gyr has led to a progressive depletion of incompatible elements in the upper mantle^{1,2}. A knowledge of the degree and uniformity of this mantle depletion throughout Earth history is important for understanding the growth of continents, the evolution of the crust-mantle system and the nature of mantle convection through time. Here we report initial ¹⁴³Nd/¹⁴⁴Nd ratios for 1.8-Gyr-old mafic volcanics from the Harts Range meta-igneous complex of central Australia which are the highest yet reported for Proterozoic igneous rocks ($\epsilon_{Nd} = +6.9$ to $+8.2$ for the least contaminated samples). These ratios far exceed those proposed in models³⁻⁵ for the isotopic evolution of the depleted mantle at this time, and imply the existence of a mantle reservoir that had been highly depleted for at least 1 Gyr. This provides strong evidence that major periods of continental growth, such as that in the late Archaean, produced long-lived heterogeneities in the upper mantle.

Neodymium isotope data were collected from tholeiitic meta-volcanic rocks of the Harts Range meta-igneous complex (HRMC) which comprises several concordant amphibolite bodies (up to 2 km thick) extending for more than 60 km within an early Proterozoic supracrustal sequence of the eastern Arunta Inlier (Fig. 1). The Harts Range sequence has been interpreted on field and geochemical grounds to represent a failed latitudinal ensialic rift⁶⁻⁹. Its minimum age is constrained by an emplacement age of 1,748 $^{+4}_{-2}$ Myr (ref. 10) for the major intrusive (Bruna) granitoid gneiss.

The HRMC tholeiites show a wide range of ϵ_{Nd} values, from $+8.2$ to $+2.8$ (Table 1, Fig. 2). Systematic isotope and geochemical differences exist between rocks from the lower and upper parts of the volcanic succession. Metatholeiites from the basal 1 km of the HRMC sequence are depleted in light rare earth elements (LREE), and have uniformly high ϵ_{Nd} values of $+8.2$ to $+6.9$. By contrast, metatholeiites from higher in the

sequence are LREE-enriched, with variable but lower $\epsilon_{\text{Nd}} = +6.3$ to $+2.8$. Regression analysis of Sm-Nd isotope data for the HRMC mafic rocks treated as a single population yields a very imprecise age of $2,176 \pm 270$ Myr with $\epsilon_{\text{Nd}}(T) = 7 \pm 2$. The scattering of the data (mean square of weighted deviations is 6.4) is greatly in excess of the experimental uncertainty, and this excess is attributed to variations in initial $^{143}\text{Nd}/^{144}\text{Nd}$. Rare-earth-element patterns and chemical characteristics are consistent with a predominantly pre-metamorphic origin, requiring involvement of pre-existing sialic material in the genesis of the upper HRMC magmas. These have lower Zr/Nb, Sm/Nd and Ti/Yb, and higher SiO_2 , K/P and Ba/Nb than the lower tholeiites, with variations in these parameters correlating with changes in $\epsilon_{\text{Nd}}(T)$. The extent of the isotope variation suggests that the contaminant was probably LREE-enriched Archaean crust that had evolved to more negative ϵ_{Nd} values. Here we consider the origin of the exceptionally high $\epsilon_{\text{Nd}}(T)$ in the least-contaminated lower HRMC tholeiites and its implication for mantle evolution. The initial ϵ_{Nd} of the HRMC samples demonstrates that this portion of early Proterozoic crust was not derived from primitive or pristine mantle, but from mantle that had an extended history of elevated Sm/Nd ratio—a characteristic of parts of the mantle that have been depleted because of extraction of continental crustal components. The high ϵ_{Nd} values for the lower HRMC tholeiites are significantly more positive than the highest ϵ_{Nd} values ($+3.3$ to $+6.5$) reported for 1.7–1.9 Gyr volcanics from southwest North America, Greenland, Finland and Canada^{11–14}. These highly positive values for the HRMC are in fact similar to modern oceanic island arcs¹⁵ and imply a long-term highly depleted source for the HRMC rocks. The HRMC ϵ_{Nd} values are substantially more depleted than that previously proposed for depleted mantle evolution^{3–5}. This type of heterogeneity, if preserved in the modern mantle, would result in mid-ocean-ridge basalts (MORB) having substantially more positive values ($\epsilon_{\text{Nd}} \approx +17$ to $+20$) than is actually observed ($\epsilon_{\text{Nd}} \approx +10$ to $+12$; ref. 16).

The Sm/Nd enrichment factor ($f^{\text{Sm/Nd}}$) of the HRMC magma source can be calculated as a function of the time, T_s , at which it became fractionated relative to its mantle source, by using the relation

$$\epsilon_{\text{Nd}}(T) = f^{\text{Sm/Nd}} Q_{\text{Nd}}(T_s - T) + \epsilon_{\text{Nd}}(T_s) \quad (1)$$

where $Q = 0.0251 \text{ Myr}^{-1}$, $\epsilon_{\text{Nd}}(T) = +8$, $T = 1,760$ Myr (emplacement age) and $\epsilon_{\text{Nd}}(T_s)$ is the Nd isotope composition of the source at time T_s . We also define the enrichment factor of the magma relative to its source:

$$\text{EF} = \frac{(\text{Sm/Nd})_{\text{melt}}}{(\text{Sm/Nd})_{\text{source}}} \quad (2)$$

This relation can be used to determine enrichment factors of the magmas relative to the proposed HRMC sources for f values calculated from equation (1).

Evidence that some mantle reservoirs experienced depletion before the end of the Archaean is indicated by positive ϵ_{Nd} values for Archaean gneisses, for example Nulliak and Uivak gneisses, Labrador¹⁷. In Australia, well-defined initial $^{143}\text{Nd}/^{144}\text{Nd}$ of Kambalda volcanics corresponding to $\epsilon_{\text{Nd}} = +4$ at 2.7 Gyr (refs 4, 18) provides definitive evidence for the existence of long-lived heterogeneities with $f > 0$ in the Archaean mantle. The Kambalda data also straddle the depleted mantle (DM) evolution curves^{4,5} in Fig. 2, and are coincident with them at $\epsilon_{\text{Nd}} \approx +4$ at 2.7 Gyr. For this reason, in our initial calculations we choose the ϵ_{Nd} and age of the Kambalda source as representing possible $\epsilon_{\text{Nd}}(T_s)$ and T_s for the HRMC source at the time that it underwent fractionation from its precursor Archaean depleted mantle. Hence, for $T_s = 2.7$ Gyr, $\epsilon_{\text{Nd}}(T_s) = +4$, $T = 1.76$ Gyr (HRMC emplacement age), $\epsilon_{\text{Nd}}(T) = +8$ (HRMC basalts), we obtain $f^{\text{Sm/Nd}} = 0.169$. This is a minimum value and implies a mantle source with a time-averaged Sm/Nd from 2.7 Gyr to 1.76 Gyr that was 17% higher than in the chondritic

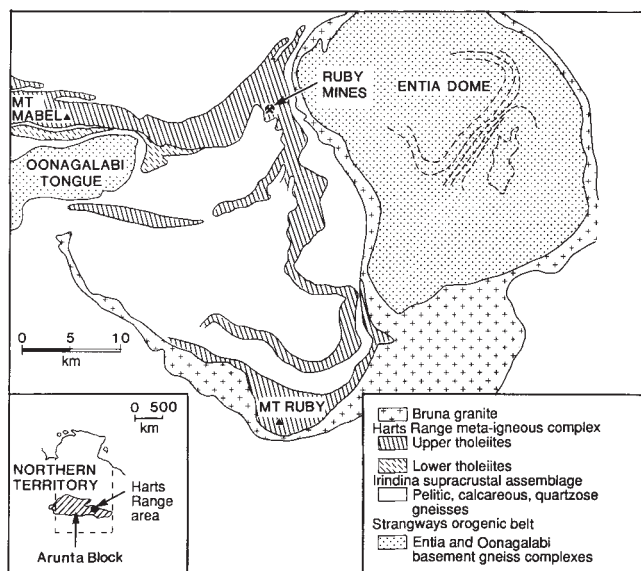


FIG. 1 Geological map of Harts Range area, central Australia, showing Harts Range meta-igneous complex. Also shown are the Irindina supracrustal cover assemblage and the Strangways orogenic belt basement gneiss complexes, with the Bruna granite intruding along their tectonic contacts.

unfractionated reservoir (such a mantle source would today have $\epsilon_{\text{Nd}} > +17$). According to equation (2), for $f^{\text{Sm/Nd}} = 0.169$, then $\text{EF} = 0.857$, which implies a 14.3% enrichment of the Sm/Nd ratio of the HRMC basalts relative to their source. The degree of partial melting ($\sim 10\%$) of the HRMC mantle source

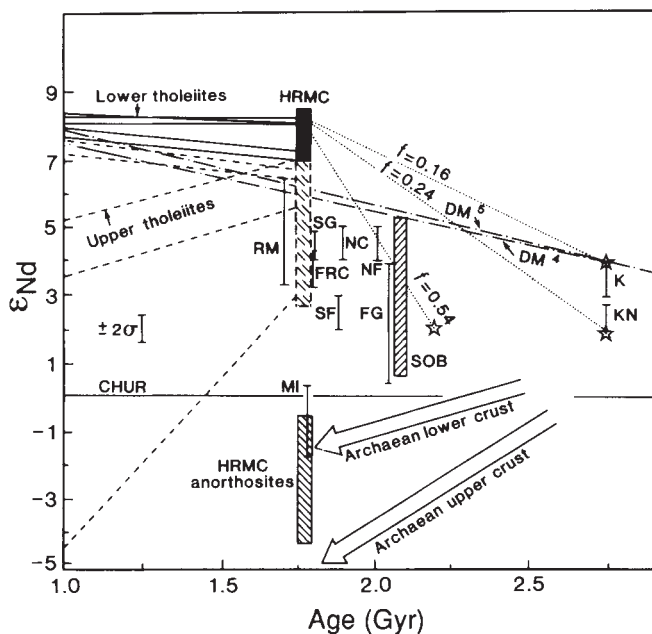


FIG. 2 Diagram of ϵ_{Nd} against age showing isotopic evolution of Harts Range meta-igneous complex (HRMC) lower (solid symbols) and upper (dashed symbols) tholeiites together with inferred trajectories (dotted lines) for their possible mantle sources, with a range of different $^{147}\text{Sm}/^{144}\text{Nd}$ enrichment factors ($f^{\text{Sm/Nd}}$). Depleted mantle evolution trends^{4,5} and $\epsilon_{\text{Nd}}(T)$ for whole-rock samples from other late Archaean and early (or mid-) Proterozoic mantle-derived magmatic suites are shown. The lower HRMC have significantly higher $\epsilon_{\text{Nd}}(T)$ values than previously recognized. Sources of data: RM, Rocky Mtns¹¹; FRC, Front Range, Colorado³; K, Kambalda⁴; KN, Kanowna⁴; NC, northern Canada²⁴; NF, northern Finland³⁰; FG, French Guiana³¹; MI, Mount Isa²⁷; SF, southern Finland¹³; SG, southern Greenland¹²; SOB, Strangways orogenic belt. Error bar shows maximum total procedural analytical uncertainty for HRMC Nd-isotope determinations.

TABLE 1 Sm-Nd isotope data

Sample	Sm (p.p.m.)	Nd (p.p.m.)	$^{147}\text{Sm}/^{144}\text{Nd}$	$^{143}\text{Nd}/^{144}\text{Nd}$	$\epsilon_{\text{Nd}}(T)^*$
HRMC lower metatholeiites					
L2	5.71	17.33	0.1991	0.513088 ± 8	8.0
G79	4.71	14.41	0.1978	0.513066 ± 8	7.9
G80A	4.81	14.78	0.1967	0.513067 ± 7	8.2
1000V	5.13	15.21	0.2038	0.513101 ± 8	7.2
G73X	4.39	12.98	0.2048	0.513095 ± 7	6.9
HRMC upper metatholeiites					
27012B	3.15	9.27	0.2055	0.513075 ± 7	6.3
27009A	4.03	13.83	0.1762	0.5126984 ± 6	5.5
R1	3.00	10.13	0.1790	0.512798 ± 7	6.9
J390003	2.39	7.04	0.2051	0.513085 ± 14	6.6
27001A	6.32	22.02	0.1736	0.512524 ± 11	2.8
27009B	4.84	24.59	0.1190	0.511909 ± 15	3.1

* $\epsilon_{\text{Nd}}(T)$ calculated for HRMC emplacement age $\sim 1,767$ Myr.

The procedures used for the isotope analyses of Sm and Nd are described elsewhere²⁸. The $^{143}\text{Nd}/^{144}\text{Nd}$ ratio of La Jolla standard measured at Australian National University during the course of this work is 0.511872 ± 5 . Total procedural blanks for Nd are < 300 pg. The ϵ_{Nd} values are defined as:

$$\epsilon_{\text{Nd}} = \left[\frac{(^{143}\text{Nd}/^{144}\text{Nd})_{\text{init}}}{(^{143}\text{Nd}/^{144}\text{Nd})_{\text{CHUR}}} - 1 \right] \times 10^4$$

where

$$(^{143}\text{Nd}/^{144}\text{Nd})_{\text{CHUR}}^T = (^{143}\text{Nd}/^{144}\text{Nd})_{\text{CHUR}}^0 - (^{147}\text{Sm}/^{144}\text{Nd})_{\text{CHUR}}^0 (e^{\lambda_{\text{Sm}} T} - 1).$$

Present-day reference values²⁹ for the chondritic unfractionated reservoir (CHUR)¹⁶ are $(^{143}\text{Nd}/^{144}\text{Nd})_{\text{CHUR}}^0 = 0.512650$ and $(^{147}\text{Sm}/^{144}\text{Nd})_{\text{CHUR}}^0 = 0.1967$; $\lambda_{\text{Sm}} = 6.54 \times 10^{-12} \text{ yr}^{-1}$; $(^{143}\text{Nd}/^{144}\text{Nd})_{\text{init}}^T$ is the measured ratio in the rock, corrected for decay since the time of crystallization T . The $^{147}\text{Sm}/^{144}\text{Nd}$ enrichment factor relative to CHUR is given by

$$f_{\text{Sm/Nd}} = \left[\frac{(^{147}\text{Sm}/^{144}\text{Nd})_{\text{meas}}}{(^{147}\text{Sm}/^{144}\text{Nd})_{\text{CHUR}}^0} - 1 \right]$$

that would be required to cause this amount of Sm/Nd fractionation is consistent with other chemical features of the HRMC tholeiites (such as their low Al/Ti and Ca/Ti ratios) which imply equivalent moderate to low percentages of mantle melting¹⁹.

We consider it unlikely that the isotope characteristics of the HRMC source could have evolved in a shorter interval of time or from a less depleted (less positive ϵ_{Nd}) late Archaean mantle precursor. The very low degrees of partial melting (2–7%) that would be necessary to produce the extreme fractionations (25–35%) in Sm/Nd in such cases (Fig. 2) would tend to generate more alkaline basalts, chemically unlike the HRMC tholeiites. Production of the early Proterozoic (residual) depleted mantle source for the HRMC basalts is most compatible with a 2.6–2.7 Gyr mantle source with $\epsilon_{\text{Nd}}(T_s) = +4$ and $f_{\text{Sm/Nd}} = 0.17$. The age of 2.6 Gyr is a reasonable minimum estimate. This is because the low $f_{\text{Sm/Nd}}$ values of possible sources resulting from older depletions do not require large Sm/Nd fractionations to generate the REE distributions of the HRMC tholeiites, and can be readily attained at moderate degrees (9–10%) of partial melting. The HRMC source proposed above could represent the residuum after extraction of late Archaean tonalites.

A predominantly asthenospheric source is proposed for the high- ϵ_{Nd} HRMC rocks. Chemical features of the least-contaminated HRMC tholeiites typify back-arc basin basalts^{21,22}: these features include ratios of Nb/Th (8–12), Th/Pb (0.17–0.34), Nb/Pb (1–4) and La/Yb (~ 1.5) which are intermediate between MORB and island arc tholeiites (IAT)²⁰, low Ba/La (2.5–4.0) and Ba/Sr (0.13–0.17) relative to IAT^{21,22}, and MORB-like Sr/Nd (~ 10), Ce/Pb (11–37), Hf/Th (> 12) and Ti/V (33–37)^{20,23}. In addition, 1760 ± 2 Myr-old mafic-felsic (Entia) orthogneisses¹⁰ structurally underlying the Harts Range sequence have pronounced Cordilleran margin geochemical signatures^{24,25}, suggesting a close temporal and spatial kinship of

the HRMC with an active early Proterozoic convergent plate margin. Conclusive evidence of crustal contamination in the upper HRMC tholeiites, together with a lack of ophiolitic suites with abundant ultramafics, favour an ensialic setting. HRMC magmas were apparently emplaced in a subduction-related extensional setting, on or near continental crust, most probably in a back-arc basin behind the Entia magmatic 'arc'. The onset of asthenosphere-dominated magmatism was triggered by extension, initiated and maintained by subduction beneath a Cordilleran-style plate margin. The ultra-depleted HRMC source was a part of a 'fossil' mantle wedge. Highly depleted mantle wedge compositions have been recognised previously in some modern subduction-related settings, such as Tonga-Kermadec²⁶.

To develop its distinctive isotope features, the HRMC source must have been largely isolated from the bulk of the convecting asthenosphere from at least 2.7 Gyr (possibly coinciding with the stabilization of Archaean continental crust) until 1.8 Gyr. Furthermore, if this type of long-lived ultra-depleted mantle was preserved today, it would have ϵ_{Nd} values of +17 to +20. Apart from rare xenoliths preserved in the sub-continental lithospheric mantle, such a composition is not observed today. What factors are likely to have controlled the preservation of this type of ultra-depleted mantle and are these factors likely to have been unique to the Archaean/early-Proterozoic period? First, the ultra-depleted mantle identified in the HRMC rocks is consistent with residual mantle produced as a result of continental crust extraction. For the Australian continent, the period from 2,600 Myr to 2,700 Myr is recognised as a principal period of crustal formation²⁷. Thus, pronounced and extensive depletions are likely to have been produced in the residual mantle. The other important factor is the style of mantle convection that is likely to have been operating from ~ 2.6 Gyr to 1.8 Gyr. It has been suggested that during this interval, mantle convection was limited to the uppermost 400 km of the mantle above a strong upper-mantle transition zone². This effectively isolated deeper, more primitive asthenosphere from upper mantle that had been highly depleted by continental extraction. In northern Australia, a major crust-forming episode occurred at ~ 1.8 Gyr, part of a worldwide thermal event which probably reflected a large perturbation in upper-mantle convection. At this time, more primitive material tapped from the transition zone (400–670 km depth), would have had the effect of diluting the more depleted portions of the upper mantle. In this situation, it is unlikely that ultra-depleted portions of the upper mantle produced during the late Archaean (~ 2.7 Gyr) would survive intact much beyond the early Proterozoic. These arguments suggest that the preservation of ultra-depleted mantle sampled by the HRMC can be largely attributed to conditions unique to this period. □

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A Met-to-Val mutation in the skeletal muscle Na⁺ channel α -subunit in hyperkalaemic periodic paralysis

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HYPERKALAEMIC periodic paralysis (HYPP)¹ is an autosomal dominant disease that results in episodic electrical inexcitability and paralysis of skeletal muscle. Electrophysiological data indicate that tetrodotoxin-sensitive sodium channels from muscle cells of HYPP-affected individuals show abnormal inactivation^{2,3}. Genetic analysis of nine HYPP families has shown tight linkage between the adult skeletal muscle sodium channel α -subunit gene on chromosome 17q and the disease (lod score, $z = 24$; recombination frequency $\theta = 0$)^{4–6}, strongly suggesting that mutations of the α -subunit gene cause HYPP. We sequenced the α -subunit coding region isolated from muscle biopsies from affected (familial HYPP) and control individuals by cross-species polymerase chain reaction-mediated complementary DNA cloning. We have identified an A $\rightarrow$ G substitution in the patient's messenger RNA that causes a Met $\rightarrow$ Val change in a highly conserved region of the α -subunit, predicted to be in a transmembrane domain. This same change was found in a sporadic case of HYPP as a new mutation. We have therefore discovered a voltage-gated channel mutation responsible for a human genetic disease.

Pedigree A (Fig. 1a) shows a small portion of the HYPP pedigree used for the original linkage analysis with the human α -subunit gene⁴. Total RNA was made from a muscle biopsy from patient IV-1 (pedigree A) and from individuals with unrelated neuromuscular disorders. RNA was reverse-transcribed and regions of the sodium-channel α -subunit cDNA were amplified using polymerase chain reaction (PCR) primers derived from rat cDNA sequence⁷. Fifteen overlapping regions of 300–700 base pairs (bp) were directionally cloned into both M13mp18 and M13mp19. As HYPP is a dominant disease, the HYPP patient is heterozygous: both a normal and abnormal α -subunit gene are present. Therefore a population of both normal and abnormal α -subunit mRNAs should exist in the patient's biopsy. To ensure that both species were detected, at least 10 clones for each amplified region from the HYPP patient's cDNA were sequenced in both directions: we expected two distinct clone populations of equal frequency at a single nucleotide position for any potential mutation. Alternatively, base changes resulting from misincorporation of nucleotides by the

Taq polymerase during PCR amplification were predicted to occur only in a small fraction of clones. A minimum of four clones from control muscle were sequenced in parallel.

Sequence analysis of the complete 5,511-bp coding region for both the patient and control α -subunit cDNA (~150,000 bp sequence screened) revealed two nucleotide changes in the patient cDNA that could not be attributed to *Taq* polymerase errors. One was a G-to-T substitution resulting in a Glu-to-Asp change; the second was an A-to-G substitution resulting in a Met-to-Val change (Fig. 1b). We then investigated whether either of these base substitutions was associated specifically with HYPP or was instead a neutral polymorphism. Affected and unaffected individuals were tested for these base changes using differential hybridization (Fig. 1c). The G-to-T substitution was present in 2 of 11 unaffected individuals, and was found not to reside on the disease chromosome in the HYPP family (pedigree A; data not shown). Thus this base substitution represents a polymorphism (Glu $\rightarrow$ Asp; Fig. 2a).

The A-to-G base substitution resided on the affected chromosome in the HYPP family (Fig. 1a and c); an unaffected family member (III₁) did not have this substitution, whereas an affected sibling was heterozygous for the mutation (III₂). This same mutation was found in a sporadic case of HYPP (Fig. 1, pedigree B, individual II₁) and not in either of her parents (I₁ and I₂) (Fig. 1c). These results were verified through digestion of PCR products with the allele-specific restriction enzymes *Mae*II (cuts mutant sequence), and *Nla*III (cuts normal sequence; data not shown), and by cloning and sequencing 10 PCR clones covering this region from genomic DNA from each family member (data not shown). Non-paternity was ruled out in the sporadic case by typing for three highly polymorphic CA repeats at the dystrophin locus: the affected daughter inherited all three alleles from her father (data not shown). Moreover, the relatively rare polymorphic G $\rightarrow$ T change was found in both the father and daughter, but not in the mother. A total of 62 individuals were tested by differential hybridization: 59 were homozygous for the normal allele (A|A), and three were heterozygous (A|G), all three being affected with HYPP.

We have shown that HYPP cosegregates with the Met $\rightarrow$ Val substitution in pedigree A. Given this family alone, this substitution may be cosegregating with the disease-causing mutation. But the identification of the same Met-to-Val substitution as a *de novo* mutation in a sporadic non-familial case of HYPP (pedigree B) strongly suggests that this amino-acid change is the mutation causing HYPP.

The finding of two independent occurrences of the same mutation suggests a propensity for mutation at this site. Interestingly, the mutated site lies within a 10-bp region showing an inverted repeat, direct repeat and dyad symmetry (Fig. 2c): these repeats may be mutagenic. But sequence analysis of 10 PCR clones from genomic DNA of each of five affected individuals from additional unrelated HYPP families failed to reveal any other mutations in this region (data not shown).

The mutation occurs in the proposed S₆ transmembrane segment of domain IV (Fig. 2a), and changes an amino acid that is conserved between all sodium channels studied so far (Fig. 2b). Evolutionary conservation of this methionine suggests a crucial structural or functional role for this residue. Although no direct information has been forthcoming on the function of this S₆ domain of the sodium channel, data on the structure and function of voltage-sensitive potassium channels has begun to accumulate. The main component of functional sodium channels is a single α -subunit chain that contains four domains (I–IV; Fig. 2a), whereas potassium channels are tetramers of smaller subunits, each comparable to a single domain of the sodium channel. The S₅–S₆ interdomain of the potassium channel may be inserted into the membrane to form the ion-conducting pore of the channel^{8–10}. If the molecular topology of the potassium channel proves to be valid for the sodium channels, the mutation we have identified lies in an α -helix immediately adjacent to

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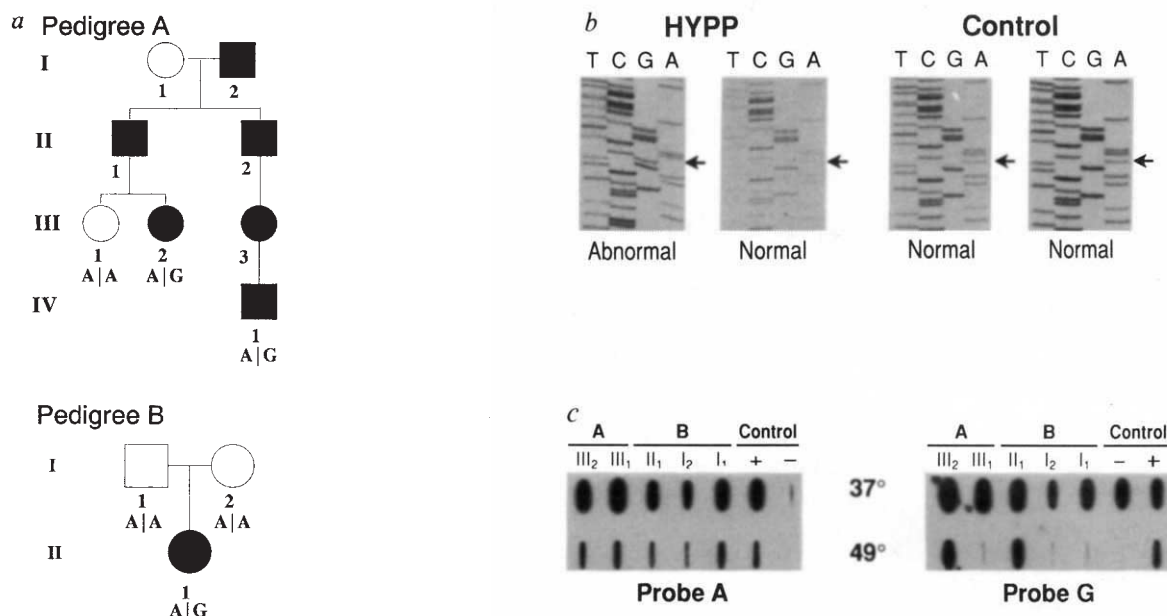


FIG. 1 Identification and verification of the A→G base substitution causing HYPP in two families. *a*, Pedigrees of the families with the HYPP mutation. Individuals tested are marked with regards to the specific nucleotide (A or G) present in each 17q sodium channel gene (A in the normal allele and G, in the HYPP mutation). *b*, An A-to-G base substitution is found in 50% of HYPP (patient IV-1) muscle α -subunit cDNA clones. The sequence analysis shown is part of the IVS₆ region representative of the two normal chromosomes (right panels) seen in unaffected individuals, and the one normal and one abnormal chromosome in the heterozygous patients with dominant HYPP. The A-to-G base change (arrows) was found only in HYPP-affected individuals in the two pedigrees shown in *a*, and not in 59 other individuals tested. *c*, Identification of the HYPP mutation by differential hybridization. Slot blots are shown of PCR products from genomic DNA of the individuals in pedigrees A and B (see *a*); hybridization with allele-specific oligonucleotides was at high and at low stringency. Blots were processed in parallel and exposed for the same time. Individuals on blots are identified by the numbers in the pedigrees in *a*. All unaffected individuals are homozygous for the normal allele and all HYPP patients shown are heterozygous (probe A; left panel). Only the affected patients are heterozygous for the mutant allele (probe G; right panel).

METHODS. PCR-mediated cross-species cDNA cloning has been described⁴. A detailed description of the cloning and sequencing strategy, the complete sequence of the normal human sodium channel α -subunit coding sequence, and the genomic structure of the gene will be published elsewhere (J.W.,

C.V.R., J. Z. Zhou and E.P.H., manuscript in preparation). Because we used cross-species cloning, both the N-terminal and C-terminal sequence (each ~20 bp) were not determined in either the patient or normal human. For differential hybridization, PCR primers (20-mers) were synthesized which flanked the base changes found by M13 chain-termination sequence analysis. The primer sets used for amplification of the HYPP mutation were as follows: forward primer, 5'-GCATCTGCTTCTCTGCAGC-3'; reverse primer, 5'-CTCGTGCTCTCTCTGTGG-3'. These primers (100 ng) were used on genomic DNA (200–500 ng) from affected and unaffected individuals in PCR (30 cycles at 94 °C for 1 min then 65 °C for 4 min). PCR products were visualized by ethidium bromide staining of 1% agarose/2% NuSeive agarose mixed gels, then roughly equal amounts were denatured and slot-blotted onto nylon membranes. Membranes were baked and processed for differential hybridization with oligonucleotides¹⁹. Oligonucleotides used for differential hybridization were (amino-acid number refers to position in rat sequence⁷): Probe A, normal allele, 5'-GGTCAACATGTACATCG-3'; Probe B, HYPP allele, 5'-GGTCAACGTGTACATCG-3'. Oligonucleotides were desalted on NAP-10 columns, and 10 pmol end-labelled using T4 polynucleotide kinase and γ -³²P-labelled ATP (10 pmol of 6,000 Ci mmol⁻¹). Labelling reactions were used directly for hybridization. Duplicate filters were washed at low stringency (0.1 × SSC, 0.5% SDS at 37 °C) and high stringency (0.1 × SSC, 0.5% SDS; at 49–50 °C), autoradiographed, and the high-stringency and low-stringency signals compared for each sample.

the pore-forming segments¹¹ and may therefore disrupt the structure or function of the pore. It is relevant that a neutral amino-acid substitution in the S₆ segment of the *Shaker* potassium channel has been shown to alter slow (C-type) inactivation dramatically¹².

How can a single conservative amino-acid change in the 1,836-amino-acid α -subunit cause the K⁺-induced failure of inactivation seen in the muscle of HYPP patients^{2,3}, and why does the mutation have a dominant effect? The Met→Val substitution is in a region of the channel which has not been directly implicated in inactivation: protease, antibody, and molecular mutation studies have instead implicated the intracellular inter-domain III–IV^{13,14}. But some factors alter inactivation at other regions of the protein. For example, scorpion α -toxins inhibit inactivation extracellularly¹⁵. At this point it is impossible to say whether the Met→Val substitution is affecting inactivation directly or indirectly. The dominant nature of the disorder is

due to the aberrant gating of the mutant channels: Hodgkin and Huxley modelling shows that a small proportion (<5%) of non-inactivating sodium channels will depolarize the cell and thereby inactivate both mutant and normal channels (S. C. Cannon, R.H.B. and D. P. Corey, manuscript in preparation). This constitutes a novel mechanism for autosomal dominant expression of a mutation, and illustrates how an environmental factor (extracellular K⁺) can modulate the expression of the mutation.

All HYPP families studied so far have shown linkage to the α -subunit gene of the adult skeletal muscle sodium channel, suggesting that it is a genetically homogeneous disease^{4–6}. The mutation that we have identified was not found in five other HYPP families, suggesting that different mutations can cause the same disease. Paramyotonia congenita, a dominant disease characterized by cold-induced hypercontraction of muscles, is also tightly linked to the α -subunit gene^{16,17}. The identification

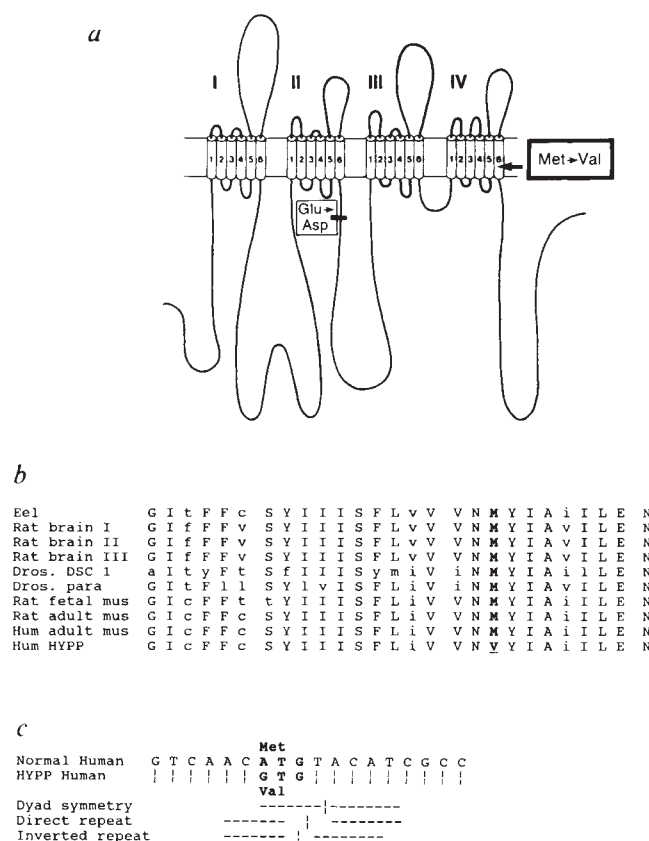


FIG. 2 Location of the HYPP mutation. **a**, The hypothetical arrangement is shown of membrane-spanning regions and intracellular (lower) and extracellular (upper) domains of the α -subunit. The HYPP mutation changes a methionine residue to a valine in the S₆ region of domain IV. The polymorphism that is not associated with HYPP changes a glutamic acid to an aspartic acid in the II-III interdomain. The diagram is modified from ref. 9. **b**, Amino-acid sequence alignment of sequenced sodium channel α -subunits over the IV S₆ region containing the HYPP mutation. The methionine shown in bold is completely conserved among all sodium channels but is mutated to a valine in one extended HYPP family and one isolated case (Fig. 1). References are as follows: eel²⁰, rat brain I and II (ref. 21), rat brain III (ref. 22), *Drosophila* DSC 1 (ref. 23), *Drosophila para* (ref. 24), rat fetal muscle²⁵, rat adult muscle⁷. Normal human adult muscle and HYPP adult muscle sodium channel sequences are from this work. **c**, Dyad symmetry, direct repeat and inverted repeat at the site of mutation: a possible propensity for mutation.

of additional α -subunit mutations causing HYPP and paramyotonia congenita will permit structure/function correlations in skeletal muscle sodium channels that will complement *in vitro* mutagenesis studies of voltage-sensitive channels¹⁸. □

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Intraclonal generation of antibody mutants in germinal centres

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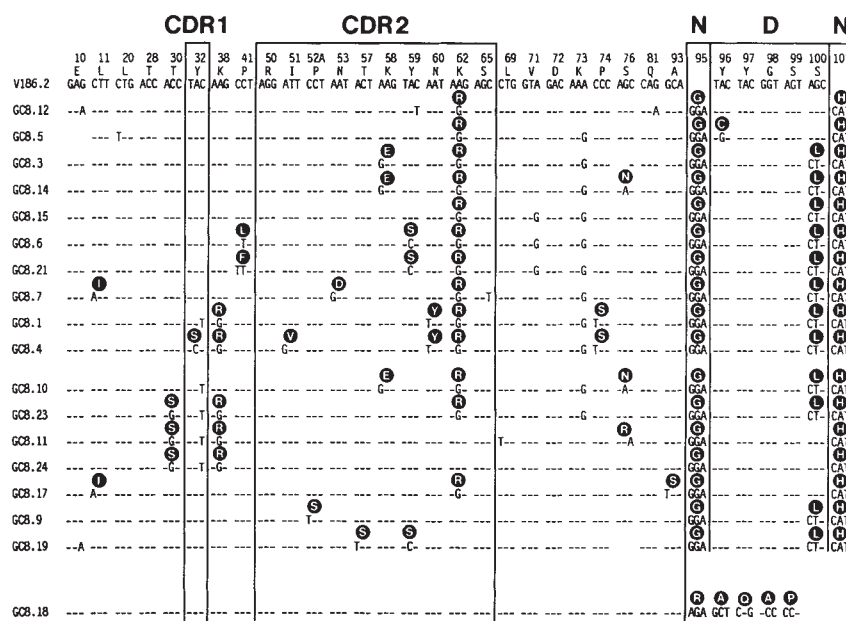
THE generation and selection of somatic antibody mutants are key elements of acquired immunity, essential for the affinity maturation of antibody responses dependent on T cells. The mutants are generated through a mechanism that introduces point mutations at high rate into rearranged variable (V) region genes in the course of cell proliferation^{1,2}. Their appearance coincides with the generation of germinal centres, which are characterized by oligoclonal B-cell proliferation^{3,4} and have been suggested to be the microenvironment in which antibody mutants are generated^{5,6}. We report here direct evidence for this hypothesis. Rearranged V-region genes were amplified from the genomic DNA of cells picked from individual germinal centres. The sequence analysis of these genes revealed that most represent cells of distinct B-cell clones which expanded locally, generating somatic antibody mutants at high rate. By contrast, antigen-induced proliferation of B cells at another site, periarteriolar lymphocyte sheath-associated foci, was not associated with somatic hypermutation.

The response to the hapten (4-hydroxy-3-nitrophenyl)acetyl (NP) coupled to protein carriers is dominated in *Igh^b* mice by antibodies which have the $\lambda 1$ light (L) chain and use heavy (H) chain V regions encoded by the V186.2 *V_H* gene segment, the FL16.1 D element and any of four *J_H* genes, with *J_H2* being most common⁷⁻¹⁰. The *V_H* to D boundary of these antibodies is characteristically a series of three tyrosines, the first of which is occasionally substituted by a glycine^{7,9}. We have used the polymerase chain reaction (PCR) to amplify H-chain V region DNA of B cells picked from individual germinal centres and of periarteriolar lymphocyte sheath (PALS)-associated foci from C57BL/6 mice immunized with NP. Such B cells can be identified in histologic sections of frozen spleen by specific immunolabelling with antigen, anti- $\lambda 1$ antibody, or for germinal centre B cells, the lectin peanut agglutinin (PNA)¹¹. Amplified DNAs representing V186.2 to *J_H2* rearrangements were cloned into plasmid vectors and the V-region inserts of individual bacterial colonies were sequenced. Figures 1 and 2 depict the sequences obtained from B cells populating two germinal centres, GC8 and GC24, 16 days after immunization. The GC8 sequences result from the amplification of DNA from a single section whereas those of GC24 were derived from two adjacent serial sections which were processed and amplified separately. Each of these germinal centres contained V186.2 genes belonging to a single lymphocyte clone as judged by their *V_H* to D and D to *J_H* junctions. Both sets of clonally related genes are heavily mutated and carry shared as well as unique nucleotide exchanges. The average number of mutations among the related sequences of GC8 and GC24 are 5.6 and 7.0, corresponding to

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FIG. 1 Nucleotide sequences of V gene segments derived from the day 16 germinal centre GC8. Only those codons that differ from the germline V186.2 and DFL16.1 are shown; dashes, identity to the reference sequence, blanks, uncertainty. Codons are numbered according to Kabat *et al.*²³ and the amino-acid substitutions are marked in black circles. Sequences GC8.12 to GC8.4 can be fitted into a genealogical tree (Fig. 3) and sequences GC8.10 to GC8.19 are classified as gene hybrids.

METHODS. A frozen spleen section from a 10-week-old female C57BL/6 mouse (immunized 16 days earlier with NP coupled to chicken γ -globulin⁴) was stained with a biotinylated anti- λ 1 antibody, LS136, followed by streptavidin fluorochrome⁴. λ 1⁺ germinal centres were identified by fluorescence microscopy and λ 1⁺ cells from individual GC were recovered using a micromanipulator-controlled micropipette. Isolated cells were transferred to a microcentrifuge tube, boiled in H₂O at 95 °C for 10 min and digested with 10 μ g proteinase (final concentration 0.4 μ g μ l⁻¹) for 2 h at 55 °C followed by a 10 min incubation at 96 °C to inactivate proteinase K. This crude lysate was subjected to two rounds of PCR using pairs of nested primers. The first round consisted of 50 cycles using previously described primers¹⁹. 1/50 of the reaction mixture was reamplified for an additional 35 cycles. The 5' primer was complementary to the initial 20 nucleotides of the V186.2 gene encoding the mature protein and in addition contained recognition sequences for the restriction enzymes *Xba*I and *Eco*RI (5'-TCTAGAAATTCAGGTCCAAGTGCAGCC-3'). The 3' primer bound to DNA sequences in the J_H2 gene segment and specified an additional *Bam*HI recognition sequence at its 5' end (5'-ACGGATCCTGTGAGAGTGGTGCCT-3'). Each cycle consisted of 96 °C for 1.4 min and 70 °C for 2 min.



Amplified DNA was purified as described¹⁹, digested with *Pst*I and *Bam*HI and inserted into pUC19. After transformation of DH-5 α , bacterial colonies were screened with a ³²P end-labelled oligonucleotide corresponding to the predominant V_H-D border (5'-GTATCTTCGAGAATAATA-3') as described¹⁹. V_H genes were sequenced by direct plasmid sequencing using the Sequenase TM kit (USB Corporation).

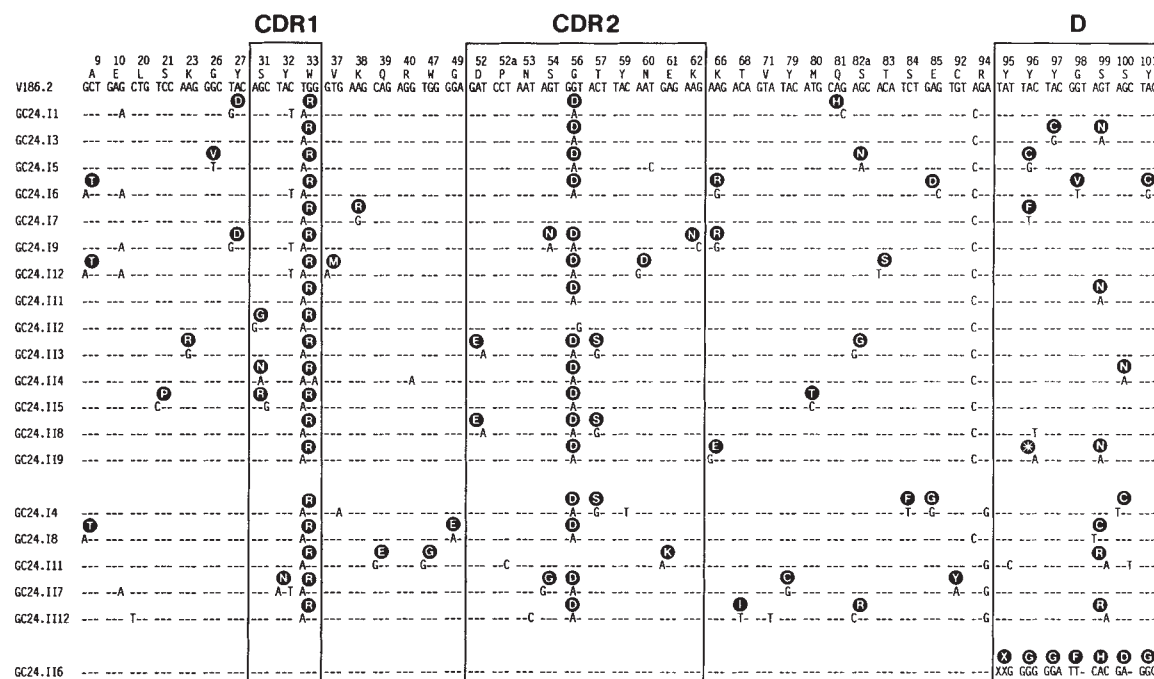


FIG. 2 Nucleotide sequence of V gene segments derived from the day 16 germinal centre GC24. Sequences indicated with the prefix I and II, respectively, are derived from the same λ 1⁺PNA⁺ GC on adjacent sections. Sequences GC24.I1 to GC24.I19 can be fitted into a genealogical tree (Fig. 3) and sequences GC24.I4 to GC24.I12 are classified as gene hybrids. X, Uncertainty; star (in GC24.I19), translational stop codon. For further description see legend to Fig. 1.

METHODS. Two adjacent frozen spleen sections were stained in tandem with PNA-alkaline phosphatase and anti- λ -peroxidase as described⁴ and examined by light microscopy. The same λ 1⁺PNA⁺ GC was recovered from adjacent sections by micromanipulation and processed independently. Iso-

lated cells were digested overnight with 10 μ g proteinase K at 37 °C. After inactivation of proteinase K (98 °C for 10 min) the lysate was amplified by two rounds of PCR using the nested primers described in Fig. 1. The first round consisted of 40 cycles (96 °C for 1.4 min and 70 °C for 3 min) and 1/25 of the reaction mixture was reamplified for an additional 40 cycles (96 °C for 1.4 min and 70 °C for 2 min). Amplified DNA was digested with *Pst*I and *Bam*HI and cloned into the plasmid vector pBluescript II SK (pBSK). After transformation, bacterial colonies were screened for V186.2 gene inserts with a ³²P end-labelled oligonucleotide (5'-CTGGAGGGTTTGTCT-3') corresponding to the V186.2 codons 71–76. Positive clones were sequenced by direct plasmid sequencing using Sequenase.

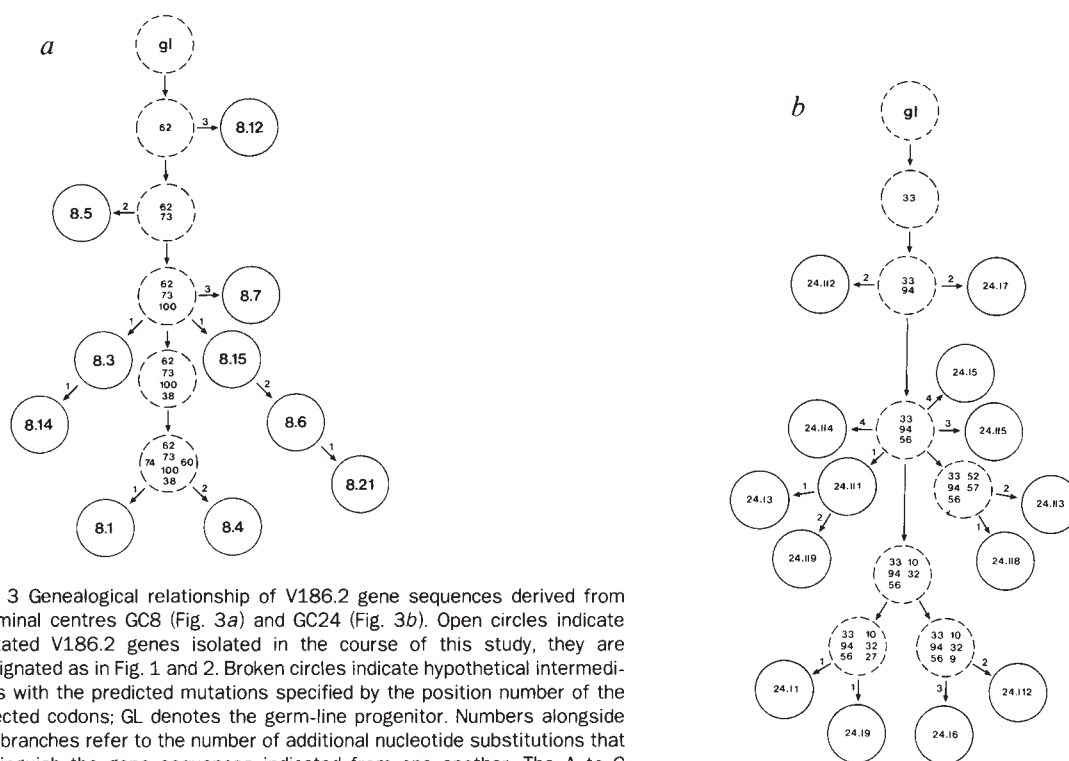


FIG. 3 Genealogical relationship of V186.2 gene sequences derived from germinal centres GC8 (Fig. 3a) and GC24 (Fig. 3b). Open circles indicate mutated V186.2 genes isolated in the course of this study, they are designated as in Fig. 1 and 2. Broken circles indicate hypothetical intermediates with the predicted mutations specified by the position number of the affected codons; GL denotes the germ-line progenitor. Numbers alongside the branches refer to the number of additional nucleotide substitutions that distinguish the gene sequences indicated from one another. The A to G exchanges at position 66 in the gene sequences GC24.I6 and GC24.I9 are assumed to have arisen as parallel independent events.

a misincorporation frequency of 1 in 56 and 1 in 45 nucleotides, respectively. By contrast, among 3,950 sequenced nucleotides representing 14 V186.2 genes derived from three $\lambda 1^+$ foci, we observed only six mutations, corresponding to a nucleotide exchange frequency of 1 in 658 (data not shown).

Although some of the substitutions found probably reflect errors of the Taq polymerase, this potential artefact is unlikely to account for the majority of the observed mutations. (1) Analogous amplification of cloned hybridoma cells suggested that the frequency of artefactual mutation (eight substitutions in 2,706 nucleotides) was low compared with that in germinal centres but similar to the exchange frequency in foci. Notably, from each germinal centre cell preparation we obtained a V186.2 gene rearranged to a *D* element atypical of the anti-NP response (presumably from contaminating B cells in the preparations) and both of these genes are unmutated. (2) The spectrum of the nucleotide exchanges does not reflect common PCR errors. The most common Taq polymerase error, misincorporation of a G opposite a template T (ref. 12), results in a preponderance of T to C and A to G exchanges^{13,14}; only about 30% of the unique exchanges observed in the V region DNA derived from GC8 and GC24 fall into this category (GC8: 8/29; GC24: 19/60). (3) Clonally related genes with shared and unique mutations were obtained when independent isolates of a single germinal centre (GC24) were separately amplified.

Thus, we believe the mutational patterns reflect the intraclonal generation of antibody mutants during the expansion of individual B-cell clones through somatic hypermutation. This process can be depicted in the form of genealogical trees¹⁵ into which we can fit 10 of the 17 related sequences originating from GC8 and 14 of the 19 related sequences originating from GC24 (Fig. 3). Some of the remaining sequences could also be incorporated in the respective tree if one assumes parallel independent mutations¹⁶. Others presumably represent gene hybrids generated during *in vitro* amplification as a consequence of processing-induced breaks. Damaged DNA templates promote the formation of chimaeric DNA molecules during PCR¹⁷.

Of the 10 related V186.2 gene sequences shown in the upper part of Fig. 3 only one (GC8.6) was isolated twice in the course of this study, likewise only one of the 10 gene sequences derived from section II of GC24 was isolated twice (GC24.II1). This is roughly the expected probability of a repeat when 10 genes are sampled from a population of 50 to 100 genes. Because the latter number is close to the number of cells from which the genes were isolated, the rate of somatic mutation in the expanding B-cell clones must be exceedingly high and at least in the order of one mutation per cell division, in accord with earlier calculations^{15,18}.

The pattern of mutations in this collection of genes is remarkable for several reasons. First, none of the sequences derived from GC8 and GC24 have the characteristics of affinity maturation in the anti-NP response, a tryptophan to leucine exchange in position 33 (refs. 8, 9). This exchange is present in 70% of secondary response anti-NP hybridomas and its absence suggests that the B cells of GC8 and GC24 were not destined to enter the memory compartment in which cells expressing high-affinity antibodies selectively accumulate¹⁹. If the distribution of mutations in the genes derived from GC8 and GC24 is representative for germinal centre B cells in general, then most antibody mutants present in germinal centres do not make it into the compartment of long-lived memory cells^{19,20}, presumably because of their insufficient antigen-binding affinities or on-rate constants²¹. Nonetheless, the gene sets in Figs. 1 and 2 show evidence of phenotypic selection in that replacement mutations are greatly under-represented in the framework regions. The observed *R/S* ratios (replacement to silent mutations) in framework regions 1–3 have values of 0.8:1 (GC8) and 1.0:1 (GC24), deviating from the ratio of 3.1:1 expected for random mutagenesis. Thus, expression of functional immunoglobulin is presumably a prerequisite for the cells to persist in the germinal centre environment.

Our data establish that somatic antibody mutants arise locally in germinal centres in the course of the immune response. In this microenvironment, the mutants are generated intraclonally,

through a mechanism introducing mutations in a stepwise manner at high rate¹⁵. Hypermutation seems to be confined to germinal centres as the mutation frequency in a second NP-specific B-cell population, the PALS-associated foci, is not greater than that expected for the Taq polymerase alone.

A similar process of somatic diversification has recently been described for the ileal Peyer's patch B cells of sheep²². In contrast to mutation in germinal centres, however, hypermutation in these Peyer's patch B cells represents a developmentally regulated process that occurs perinatally and is set in motion in the absence of stimulation by external antigens. □

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Formation of a nine-subunit complex by HLA class II glycoproteins and the invariant chain

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HLA CLASS II molecules are heterodimeric transmembrane glycoproteins that bind and present processed antigenic peptides to CD4-positive T lymphocytes. Intracellularly, class II molecules associate with a third subunit termed the invariant (I) chain. Here we describe the physical characteristics of the intracellular class II $\alpha\beta$ I complex. Chemical crosslinking, size exclusion chromatography and sedimentation velocity studies demonstrate that the $\alpha\beta$ I complex is a nine-subunit transmembrane protein that contains three $\alpha\beta$ dimers associated with an I chain trimer. The organization of class II α - and β -subunits in such a multimer may have a role in the documented ability of the I chain to inhibit peptide binding to class II molecules¹⁻³. In addition, the formation of the nine-chain complex may induce the structural changes necessary to overcome the cytoplasmic retention signal responsible for the localization of free I chain in the endoplasmic reticulum, releasing class II-I chain complexes for transport to endosomes⁴⁻⁶.

It was originally proposed that the $\alpha\beta$ I complex had a Stokes radius identical to that of a globular protein⁷ of relative molecular mass 270,000 (M_r 270 K) because it contained a chondroitin sulphate-modified form of the I chain⁸. However, it has since been shown that only a small fraction of the I-chain pool is modified by chondroitin sulphate^{9,10}. We therefore determined by chemical crosslinking whether the large size of the $\alpha\beta$ I complex results from the association of multiple DR α and β chains with the I chain. Highly purified, [³⁵S]methionine-labelled $\alpha\beta$ I complexes were incubated with increasing concentrations of the reducible crosslinking reagent dithiobis (succinimidyl propionate) (DSP) and the reaction products

TABLE 1 Physical properties of class II MHC molecules

Protein	R_e	$S_{20,w}$	M_r (protein-detergent)	M_r (protein)
$\alpha\beta$ dimer	45 Å	4.3S	95K	63K
I trimer	67 Å	5.9S	200K	130K
$\alpha\beta$ I complex	72 Å	11.1S	390K	260K

The R_e of $\alpha\beta$ dimers, uncomplexed I chain, and $\alpha\beta$ I complexes were determined by HPSEC using a TSK G4000SW size-exclusion chromatography column in a buffer of 50 mM sodium phosphate, 1% C₆Glc, pH 7.0, by comparison with the R_e of standard water-soluble proteins^{13,15}. The observed sedimentation coefficient for a protein in a medium m at temperature T ($S_{T,m}$) is given by the equation: $S_{T,m} = \ln(r_2/r_1) / \omega^2 t$, which relates the sedimentation coefficient for a boundary moving from r_1 to r_2 in terms of the angular velocity of the rotor (ω) and time of centrifugation, t (ref. 16). The $S_{T,m}$ values for $\alpha\beta$ dimers, uncomplexed I chain and $\alpha\beta$ I complexes were directly calculated from the data shown in Fig. 3. The observed sedimentation coefficient can be converted to the sedimentation coefficient at the 'standard state' (in water at 20 °C; $S_{20,w}$) by the equation $S_{20,w} = S_{T,m} [\eta_{T,m}(\rho_p - \rho_{20,w}) / \eta_{20,w}(\rho_p - \rho_{T,m})]$, where $\eta_{T,m}$ and $\eta_{20,w}$ are the viscosities of the medium at temperature T or water at 20 °C, $\rho_{T,m}$ and $\rho_{20,w}$ are the density of the medium at temperature T or water at 20 °C, and ρ_p is the density of the protein in solution (the reciprocal of the partial specific volume). Values of η and ρ were from standard tables of sucrose viscosity and density at various temperatures¹⁷. The $S_{20,w}$ values for bovine serum albumin, bovine catalase and mouse IgG standards were also obtained under these sedimentation conditions and were within 10% of literature values. The M_r of each protein-C₆Glc complex was calculated using the equation $M(1 - \bar{v}\rho_{20,w}) = 6\pi N_A S_{20,w} R_e$, where M is the M_r , $\bar{v}$ is the protein-C₆Glc complex partial specific volume, and N_A is Avogadro's number. As data on detergent binding to class II glycoproteins is not available, we have made the assumption that the partial specific volume of the protein-C₆Glc complexes is similar to that of other transmembrane glycoprotein-detergent complexes ($\bar{v} = 0.77 \text{ cm}^3 \text{ g}^{-1}$; refs 18, 19) and that these proteins bind 0.5 g detergent per g protein (refs 18-20). As discussed in the text, essentially identical R_e and $S_{20,w}$ values were obtained for class II molecules in detergent solutions containing either C₆Glc or DOC. As DOC has a partial specific volume which is similar to that of most glycoproteins ($\bar{v} = 0.73 \text{ cm}^3 \text{ g}^{-1}$), the effect of this detergent on partial specific volume of the protein-detergent complex is thereby minimized.

analysed by nonreducing SDS-PAGE. Figure 1 shows that with increasing concentrations of DSP there was an increase in the amount of a high- M_r complex and a decrease in free DR α , β and I chains. Similar results were obtained when B-lymphoblastoid cell line (B-LCL) lysates were treated with DSP before isolation of the $\alpha\beta$ I complexes (data not shown). The M_r of the terminally crosslinked product was estimated to be 260K. The above results were not a consequence of indiscriminate

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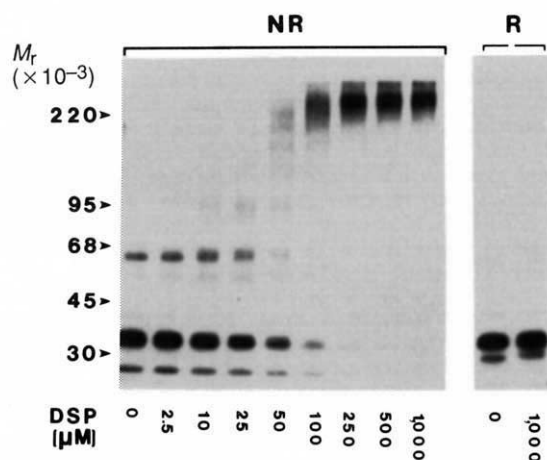


FIG. 1 Cross-linking of the $\alpha\beta$ I complex with DSP. [^{35}S]Methionine-labelled $\alpha\beta$ I complexes were incubated alone or with various concentrations of the reducible crosslinking reagent DSP. After incubation for 1 h on ice, the samples were boiled in SDS under nonreducing (NR) or reducing (R) conditions and analysed by SDS-PAGE and fluorography. (The 66K protein corresponds to disulphide-linked I chain dimers formed by the oxidation of the cysteine residue in the cytoplasmic tail of individual I chains¹¹). The M_r of the following standard proteins are indicated in the figure: carbonic anhydrase (30K), ovalbumin (45K), bovine serum albumin (68K), phosphorylase b (95K), and myosin (220K).

METHODS. The HLA-DR5 homozygous B-LCL Swei was maintained in culture as previously described¹². Class II molecules from Swei cell detergent lysates were purified by immunoaffinity chromatography and HPSEC and maintained in a buffer of 50 mM sodium phosphate, 1% *n*-octyl- β -D-glucopyranoside (C₈Glc), pH 7.0, in ice^{13,11}. Samples of various concentrations of the reducible homobifunctional crosslinking reagent DSP (dissolved in dimethyl sulfoxide) were diluted 100-fold into samples containing [^{35}S]methionine-labelled $\alpha\beta$ I complexes in a buffer of 45 mM sodium phosphate, 100 mM *N,N*-bis(2-hydroxyethyl)glycine, 1% C₈Glc, pH 8.3, on ice. After 1 h, the reaction was quenched by the addition of 1% volume of 1 M glycine. Samples were then boiled in SDS for 3 min under nonreducing or reducing conditions and analysed by SDS-PAGE using 5–10% linear acrylamide gradient gels¹². The radioactive protein present in the gels was visualized by fluorography using Kodak X-Omat AR film and Enlightening (Dupont).

crosslinking of class II subunits. Unlabelled $\alpha\beta$ dimers crosslinked with DSP and analysed by western blotting decreased in electrophoretic mobility to a position corresponding to a heterodimeric protein of 61K and DSP treatment neither revealed nor induced any higher order multimers of α and β chains. By contrast, when unlabelled $\alpha\beta$ I complexes were similarly crosslinked and analysed, the mobility of all three subunits decreased to identical positions corresponding to a complex of 270K (not shown).

Free I chain self-associates to form a trimer^{10,11}. As the association of three α and three β chains with a trimeric I chain would result in an $\alpha\beta$ I complex of about 280K, we set out to confirm that the subunits are present in equimolar amounts. The amount of [^3H]leucine present in each subunit of [^3H]leucine-labelled $\alpha\beta$ I was calculated by two-dimensional PAGE and laser densitometry⁷. An $\alpha\beta$ I complex stoichiometry of 1.0:0.9:1.3 was found for this preparation. This result agrees well with a previously published subunit stoichiometry of 1.0:1.1:0.9 (ref. 7), confirming that the three subunits in the $\alpha\beta$ I complex are present in equimolar amounts.

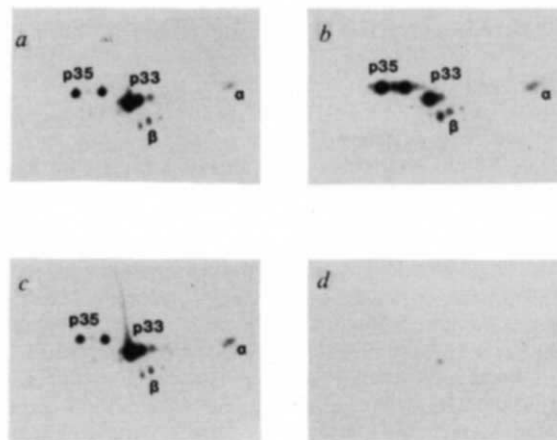
To confirm that an individual $\alpha\beta$ I complex contains multiple copies of the I chain, we took advantage of the fact that in human cells a proportion of the I chain contains a 16-amino-acid amino-terminal extension, giving rise to a 35K form of the I

chain (p35) in addition to the predominant 33K (p33) form of the I chain¹³. Anti-peptide sera directed against residues in the p35 extension or to residues in common to both p33 and p35 were generated and these antisera were used to determine whether individual $\alpha\beta$ I complexes contain both p35 and p33 forms of the I chain. The anti-p33/p35 serum (Fig. 2a) and the anti-class II serum (Fig. 2c) precipitated all of the $\alpha\beta$ I present. Figure 2b confirms that $\alpha\beta$ I precipitated by the anti-p35 serum contained both p35 and p33 forms of the I chain. These results unambiguously demonstrate that individual $\alpha\beta$ I complexes can contain more than one I chain. Similar experiments have detected both human and mouse class II β chains in $\alpha\beta$ I complexes isolated from a human B-LCL transfected with mouse *I-A^k* genes (J. M. Riberdy and P. C., manuscript in preparation). In addition, de Kretser *et al.* demonstrated that an HLA-DR-specific monoclonal antibody was capable of coprecipitating additional human class II molecules, in retrospect probably HLA-DQ, suggesting that both HLA-DR and HLA-DQ were present in the same macromolecular complex¹⁴. Taken together, these results are consistent with the hypothesis that the $\alpha\beta$ I complex contains three I chains associated with three α and three β chains.

The Stokes radii (R_e) of purified HLA-DR $\alpha\beta$ dimers, I chain, and $\alpha\beta$ I complexes were determined by high-performance

FIG. 2 Identification of different I chain forms in the $\alpha\beta$ I complex. [^{35}S]Methionine-labelled $\alpha\beta$ I complexes were treated with an anti-p35 serum (a), an anti-p33/p35 serum (b), an anti-DR serum (c), or a control serum (d) and immunoprecipitated with protein A-Sepharose. The position of the DR α and β chains as well as the p35 and p33 forms of the I chain are indicated. The immunoprecipitates were then analysed by two-dimensional PAGE and fluorography. b, Fluorograph exposed to obtain the same intensity of DR α and β chains as shown in a and c, which confirms that the p35 form of the I chain is a minority of the total class II-associated I chain pool.

METHODS. Rabbit antisera specific for HLA-DR $\alpha\beta$ dimers (R.DRAB.1), for a synthetic peptide corresponding to the 16-residue amino-terminal extension present on the p35 form of the I chain (Rlp35N), or for a peptide corresponding to amino acids 12–28 of the p33 form of the I chain (RI.EQLP) were prepared as described¹¹. Purified [^{35}S]methionine-labelled class II molecules were immunoprecipitated using 10 μl rabbit antisera and 25 μl protein A-Sepharose in a volume of 500 μl . After two washes in distilled H₂O, the immunoprecipitates were solubilized in urea, analysed by two-dimensional PAGE (nonequilibrium pH gradient electrophoresis run from right to left (acidic to basic) followed by reducing SDS-PAGE in 10% gels), and visualized by fluorography¹².



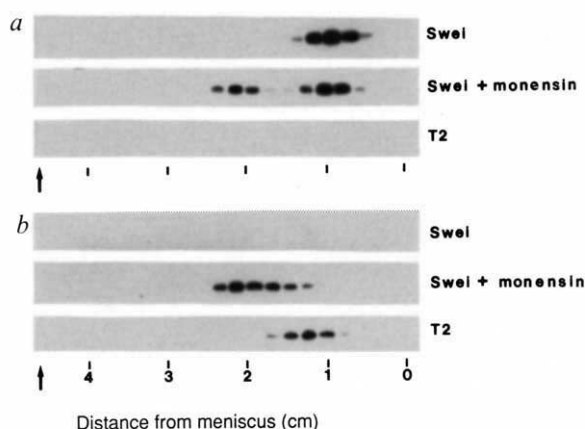


FIG. 3 Sedimentation behaviour of $\alpha\beta$ dimers, uncomplexed I chain, and the $\alpha\beta$ I complex. Cell lysates containing $\alpha\beta$ dimers (Swei), $\alpha\beta$ dimers as well as $\alpha\beta$ I complexes (Swei + monensin) and uncomplexed I chain (T2) were analysed by 5–20% sucrose gradient ultracentrifugation at 4 °C. The contents of each sample were fractionated and analysed by SDS-PAGE and analysed by western blotting with an anti-DR α chain (a) or anti-I chain (b) specific monoclonal antibody and ^{125}I -labelled second antibody. The distance of each fraction from the meniscus was calculated using the known geometry of the ultracentrifuge tube. The arrow indicates that the bottom of the centrifuge tube was 4.59 cm from the meniscus.

METHODS. Density gradient ultracentrifugation was essentially as described¹⁶. Briefly, 100 μl of detergent lysates from untreated or monensin-treated Swei B-lymphoblastoid cells (ref. 21) or untreated T2 cells (ref. 11) were carefully layered on 5–20% sucrose gradients in 50 mM sodium phosphate, 1% C_8Glc , pH 7.0 at 4 °C. After centrifugation at 35,000 r.p.m. for 16 h at 4 °C in a Beckman type SW50.1 rotor, the bottom of the centrifuge tubes were punctured and 13-drop fractions were eluted at 10 ml h^{-1} . Samples of each fraction were then analysed by SDS-PAGE and western blotting. The blots were then probed with monoclonal antibodies to the DR α chain (DA6.147; ref. 22) or to a peptide corresponding to residues 12–28 of the I chain (PIN.1.1) followed by ^{125}I -labelled OX-20 (rat anti-mouse kappa chain). The migration distance from the meniscus to the protein peak was estimated by the symmetry of a curve determined by densitometry of the blots.

size-exclusion chromatography (HPSEC) in the presence of the nonionic detergent *n*-octylglucoside (C_8Glc). The R_e of $\alpha\beta$ dimers in this detergent was 45 Å, whereas the R_e of the $\alpha\beta$ I complex was 72 Å (Table 1). This value is consistent with the previously described chromatographic behaviour of $\alpha\beta$ dimers and $\alpha\beta$ I complexes in the detergents CHAPS ((3-(3-cholamidopropyl)-dimethyl-ammonio)-1-propanesulphonate) and sodium deoxycholate (DOC)^{7,10}. In agreement with our previous studies^{10,11}, analysis of free I chain under the conditions described here revealed that the I chain/ C_8Glc complex had the rather large R_e of 67 Å. Thus the $\alpha\beta$ I complex has a R_e only 5 Å greater than that of free I chain.

Class II $\alpha\beta$ dimers, I chain and $\alpha\beta$ I complexes were also examined by sedimentation velocity ultracentrifugation. Figure 3a shows an anti- α chain and Fig. 3b shows an anti-I chain western blot of eluted fractions isolated after centrifugation of detergent lysates of lymphoblastoid cells through sucrose gradients. The calculated sedimentation coefficient ($s_{20,w}$) for $\alpha\beta$ dimers was 4.3S (Table 1). In agreement with previous results²¹, treatment of B-LCL with the ionophore monensin led to the accumulation of $\alpha\beta$ I complexes in addition to the cell-surface $\alpha\beta$ dimers (Fig. 3, middle panels). The position of the I chain-associated class II molecules can be ascertained by comparison of the middle panels of Fig. 3a and b. The $s_{20,w}$ for the $\alpha\beta$ I complex was 11.1S. The $s_{20,w}$ for $\alpha\beta$ dimers and $\alpha\beta$ I complexes were also determined in the detergent DOC, and these values varied from those obtained in C_8Glc by less than 10% (data not shown). Although not visible at the exposure shown, untreated B-lymphoblastoid cells contain small amounts of $\alpha\beta$ I complexes which also sediment at 11.1S. Free I chain sedimented at 5.4S (Fig. 3b). This relatively small $s_{20,w}$ value, as compared with the large R_e of 67 Å, suggests that the uncomplexed, trimeric I chain must either exist in an extended, asymmetrical conformation or bind anomalously large amounts of C_8Glc .

The M_r s of the different class II/ C_8Glc complexes calculated from the $s_{20,w}$ and R_e values for the $\alpha\beta$ dimer, $\alpha\beta$ I complex and I chain are shown in Table 1, as are estimates for the M_r of the protein components of each preparation. The excellent agreement of the estimate of the M_r for the $\alpha\beta$ dimer (63K) with its known M_r (62K) confirms our assumption regarding detergent binding to the $\alpha\beta$ dimer. The M_r of free I chain was estimated to be 130K. The deviation of this value from the expected value of 100K for the I chain trimer may reflect unusually high amounts of detergent binding to the I chain. The

M_r of the protein component of the $\alpha\beta$ I complex was estimated to be 260K, in excellent agreement with the value deduced from SDS-PAGE of cross linked $\alpha\beta$ I and close to the result expected for a nine-chain structure containing equal numbers of α , β and I chains.

The characterization of $\alpha\beta$ I as a large macromolecular complex has implications for the known functions of the I chain. Before $\alpha\beta$ association, the I chain remains in the endoplasmic reticulum as a trimer, a property ascribed to the cytoplasmic domain of the p35 form^{4–6}. The generation of the nine-chain $\alpha\beta$ I multimer may release the complex for transport by either masking the retention signal directly, or by inducing a conformational change in the cytoplasmic domain of the I chain. Additionally, association with the I chain inhibits peptide binding to $\alpha\beta$ dimers^{1–3}. Previously we considered two mechanisms that might explain this. Either the I chain could sterically block access of peptides to the peptide-binding groove or association with the I chain might cause a conformational alteration in the $\alpha\beta$ dimer which prevents peptide binding. A third possibility which now arises is that the overall conformation of the multimer may not allow peptide binding because individual $\alpha\beta$ dimers in the complex are so oriented that access to the peptide-binding groove is inhibited. □

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Generation of p50 subunit of NF- κ B by processing of p105 through an ATP-dependent pathway

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THE transcription factor NF- κ B is a heterodimer consisting of two proteins encoded by different members of the *rel* gene family (p50 and p65)¹⁻⁷. The p50 subunit is unusual among DNA-binding proteins in that its functional form is encoded in an open reading frame of relative molecular mass 105,000 (p105; ref. 4). The N-terminal region of this open reading frame encodes p50, whereas the remaining C terminus contains ankyrin repeats. Although p50 binds to DNA, full-length p105 translated *in vitro* does not^{4,5}. The mechanism by which p50 is generated *in vivo*, and the fate of the C-terminal region of p105 have not been established. Here we show that functional p50 is produced by ATP-dependent proteolysis of p105. Moreover, we find that the C-terminal half of p105 is not required for processing *in vivo*, and is rapidly degraded on processing. We propose that the C-terminal region of p105 is involved in the cytoplasmic assembly of the complex between the p50/p65 heterodimer and the inhibitor I κ B.

Both p50 and p105 are detected in lysates of cells labelled *in vivo* and are produced during *in vitro* translation of full-length transcripts⁸. We have confirmed this observation with HeLa cell extracts where equal amounts of p105 and p50 can be detected (data not shown). To determine whether p50 is generated by processing of p105, and to investigate the fate of the C terminus of p105, we did a pulse-chase analysis of *in vivo* labelled p105-derived proteins. The N- and C-terminal portions of the putative precursor were monitored separately using specific peptide tags^{9,10} (p97DT, Fig. 2a). COS cells transfected with p97DT were labelled briefly with ³⁵S-methionine and the lysates were subjected to immunoprecipitation using monoclonal antibodies specific for the N- or C-terminal peptide tags. Antibody

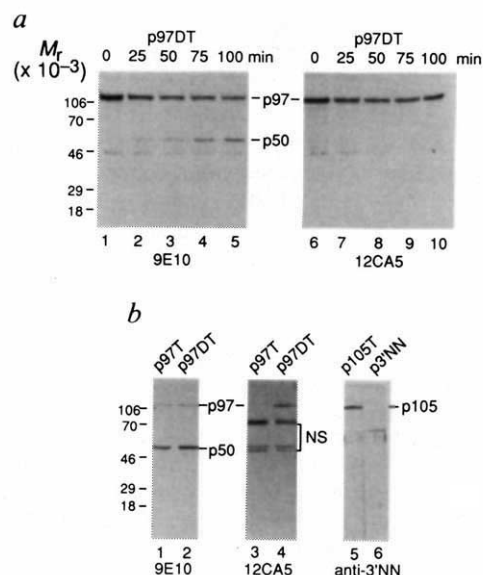
9E10, which recognizes the N-terminal tag, detected both 50K (relative molecular mass, 50,000) and 97K proteins (Fig. 1a, lanes 1-5). Most importantly, p50 increases in proportion to the decline of p97 during the chase period, indicating that p50 is generated by processing of p97 (and by implication, p105).

Significantly, no processing intermediates were identified, nor was there an increase in a C-terminal product during the chase period (Fig. 1a, lanes 6-10; Fig. 1b, lane 4). The C terminus was also not detected with polyclonal antibodies against p105 C-terminal sequences using an expression plasmid (tagged or untagged) encoding full-length p105 (Fig. 1b, lane 5). These results strongly suggest that the C terminus of p105 is rapidly degraded after the generation of p50. Thus, the C terminus must function before, but not after, p105 processing. To determine whether the C terminus is required for p50 processing *in vivo*, we tested a series of C-terminal deletions of p105 for processing in COS cells (Fig. 2). The six ankyrin repeats of p105 are not required for p50 production (Fig. 2a, p60Tth; Fig. 2b, lane 4), and the normal cleavage site of p105 seems to be near amino acid 435 (Fig. 2a, p48Nco; Fig. 2b, lane 6). The p50 molecules generated by processing of the truncated p105s in COS cells were functional in DNA binding and cotransfection assays (Fig. 3). Specific DNA-protein complexes were formed between p50 homodimers (complex Y) or the p65/p50 heterodimer (complex X) and the κ B site, or the PRDII site¹¹ of the human β -interferon promoter (Fig. 3a). The p65 homodimer does not efficiently bind either DNA probe as previously reported⁶. Virus induction of the reporter genes linked either to the κ B or PRDII sites was repressed by transfection of p105 or its deletion derivatives. But cotransfection of p65 and the p105 derivatives strongly activated transcription from either binding site (Fig. 3b). Other members of the *rel* gene family were previously shown to activate or repress transcription^{16,17}.

The endogenous p105 is evenly distributed in the cytoplasm, as indicated by immunofluorescent staining polyclonal antibodies to the C terminus of p105 (not shown), suggesting that a soluble cytosolic protease is involved in p105 processing. Both p105 and p50 are produced during *in vitro* translation in reticulocyte lysate⁸ (not shown). We have shown that an *in vitro* translated p60Tth (or a full-length p105; not shown) precursor is processed in a HeLa cell cytoplasmic extract to generate p50

FIG. 1 a, Immunoprecipitation¹⁸ of the ³⁵S-methionine pulse-labelled p97 and p50 produced in COS cells transfected with p97DT (Fig. 2a). Lanes 1-5, immunoprecipitations with 9E10 monoclonal antibody (specific for Myc tag⁹), and lanes 6-10, immunoprecipitations with 12CA5 monoclonal antibody (specific for influenza haemagglutinin (HA) tag¹⁰) (also see Fig. 2a). b, Western analyses¹⁹ of lysates of COS cells transfected with p97T, p97DT, p105T and p3'NN. Lysates were analysed on the same gel and, after transfer, the nitrocellular paper was sliced to strips for detection by different antibodies as indicated. Lanes 1 and 3 are lysates from the p97T transfectant, and lanes 2 and 4, p97DT. NS, non-specific proteins. For the right panel, p105T (lane 5) and p3'NN (lane 6) transfected COS cell lysates were probed with a mouse polyclonal antibody against C terminus of p105 (see below). Overexposure of the western blots or IP gels revealed minor polypeptides of 60-80K at ~10-fold lower amounts than the processed N-terminal p50 (not shown). These polypeptides are most probably degradation or processing intermediates of the C terminus.

METHODS. COS cells were transfected by the standard method using DEAE-dextran. Thirty hours after transfection, cells were treated with 0.1 mCi Tran³⁵S-label (ICN) on a 30 mm plate for 20 min. Cells were then washed and replenished with complete medium for the time indicated on each lane and immunoprecipitated as described¹⁸. The primary antibodies used for western blots are indicated. The secondary antibody was alkaline-phosphatase conjugated rabbit anti-mouse immunoglobulin (Sigma). The proteins were visualized by colour developing with NBT and BCIP (Sigma). As a control, the mouse polyclonal antibodies generated against the p105 C terminus (a bacterial fusion protein containing amino acids 503-777 of p105; C.M.F. and T.M., unpublished results); did recognize the COS cell produced p3'NN protein (encoding amino acids 435-880 of p105; Fig. 2a).



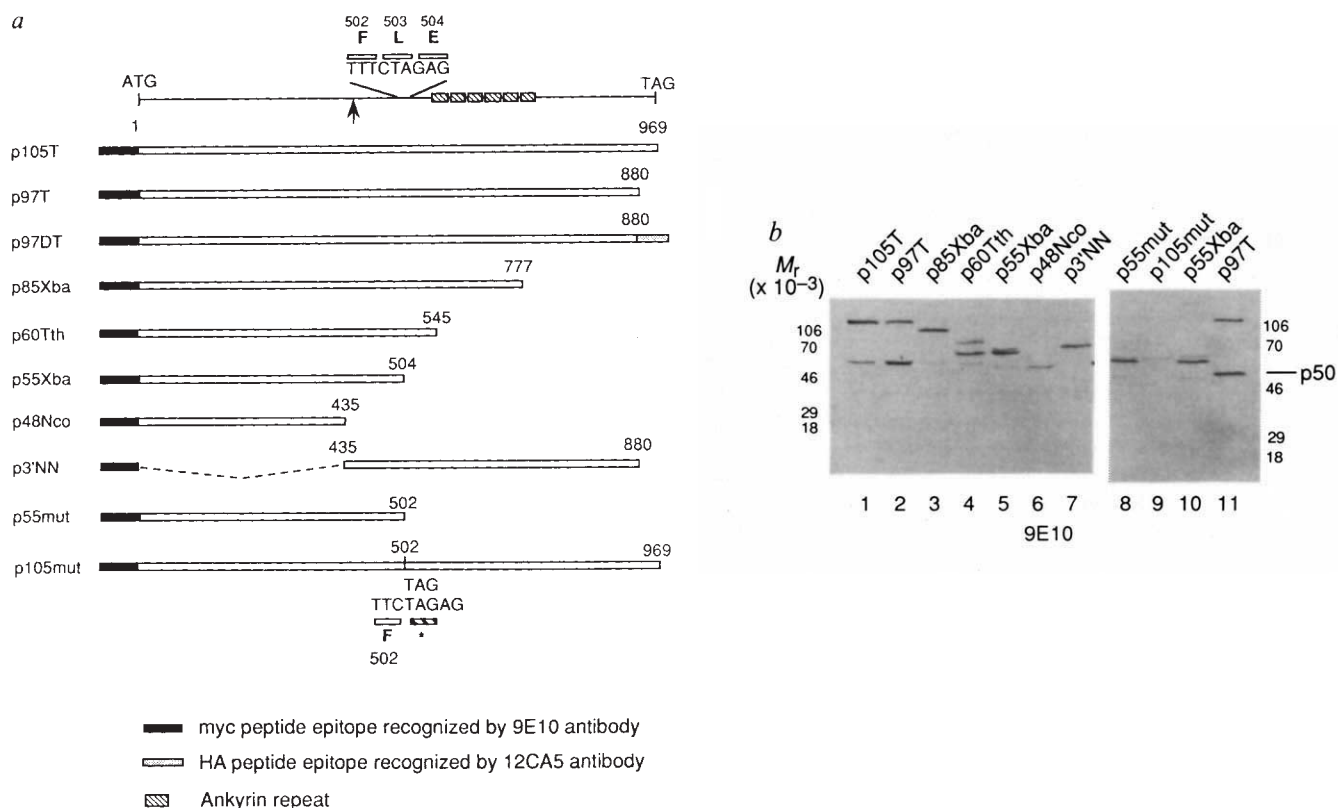


FIG. 2 *a*, Human p105 cDNA expression constructs. The approximate C terminus of p50 is indicated by an arrow. Nucleotide and amino-acid sequences of the mutated region are labelled. The N-terminal tag is a defined peptide sequence from the Myc protein⁹, and the C-terminal tag, a peptide from the influenza haemagglutinin (HA) protein¹⁰. Tagged p50 was generated from precursors having only the N-terminal 880 (p97T), 777 (p85Xba) or 545 (p60Tth) amino acids of p105. *b*, Western analyses¹⁹ of lysates from COS cells transfected with deletion plasmids: lane 1, p105T; lane 2, p97T; lane 3, p85Xba; lane 4, p60Tth; lane 5, p55Xba; lane 6, p48Nco; lane 7, p3'NN; lane 8, p55mut; lane 9, p105mut; lane 10, p55Xba; lane 11, p97T. Positions of M_r standards, p50 and a nonspecific (NS) protein are indicated. Proteins of 48K and 50K were generated from a tagged protein containing the first 435 amino acids of p105 (Fig. 3a, p48Nco; Fig. 2b, lane 6). Thus, the normal cleavage site must be located close to this region. Inclusion of 71 amino acids spanning the predicted cleavage site did not lead to the production of p50. Instead, proteins of 56K, 54K and 48K were generated from the p55Xba construct (lane 5).

METHODS. p105 cDNA were cloned into the *EcoRV* site of the expression vector pcDNA1 (Invitrogen). Deletions were generated by using convenient

restriction enzyme sites (i.e. *Tth*1111, *Xba*I and *Nco*I sites). The C-terminal sequences following the deletion positions that differ from p105 are: p97T and p3'NN: IRSITLAAARACI (single-letter amino-acid code), p85Xba and p55Xba: GPIL, p60Tth: AARACI, p48Nco: GSSMMHEGPIL. The Myc tag (a *Hind*III-*Eco*RI fragment) was fused to the N terminus of every expression plasmid. p97DT was truncated at its C terminus to fuse to the HA tag for monitoring the fate of the processed C terminus. The two isogenic expression plasmids p105mut and p55mut differed in mRNA but not in peptide sequence: nucleotide 1,506 of the p105 cDNA coding region was deleted, and a UAG stop codon was thus created at the position of amino acid 503. The p105mut plasmid contained all the coding sequence following the UAG codon, whereas p55mut did not. p105mut produced only the predicted 55K protein *in vivo* (this result also argued against the translation termination due to specific mRNA sequences), whereas p55mut (as in the case of p55Xba) produced the aberrant 54K and 48K proteins in addition to the 55K protein (*b*, lanes 9 and 10). Thus, it appears that these products result from aberrant translation of the altered mRNA sequence rather than aberrant processing. The short polypeptides produced by p60Tth, and p55Xba may occur for the same reason.

(Fig. 4a). Consistent with the data obtained *in vivo*, the C terminus cannot be detected after processing *in vitro* (not shown). We also find that p105 processing activity is dependent on ATP/Mg²⁺, and is inhibited by haemin, EDTA, and N-ethylmaleimide but not by the protease inhibitors phenylmethylsulphonylfluoride (0.2 mM) or aprotinin (1 μ g ml⁻¹). Although all of these characteristics are shared by the ATP-dependent protease complex of the ubiquitin-mediated protein degradation pathway^{13,14}, additional studies will be required to determine whether this pathway or another ATP-dependent protease is involved in p105 processing. The accuracy of the *in vitro* processing is indicated by the observation that the *in vitro* degraded tagged p50 (Fig. 4b, lane 10) has the same mobility on a SDS-PAGE as the tagged p50 produced in COS cells (lane 8), and is 2K (as predicted) larger than the untagged p50 produced in COS (lane 2) and HeLa cells (lane 6) and *in vitro* (lane 4).

The results presented here show that functional p50 is produced by ATP-dependent processing of p105, and that the

ankyrin repeats located in the C-terminal region of p105 are not required for accurate processing. Moreover, the C-terminal region of the protein is rapidly degraded as p50 is generated. A function for the C terminus of p105 before processing is suggested by the 65% sequence similarity between the ankyrin repeat regions of p105 and I κ B (ref. 15), the cytoplasmic inhibitor of NF- κ B (ref. 3). These ankyrin motifs may target p105 and p65/I κ B to a common cytoplasmic anchor, thereby aiding the assembly of a p50/p65/I κ B ternary complex after processing. We imagine dual functions of p50: p50 produced and associated with the p65/I κ B complex is a cytoplasmically localized transcription activator poised for induction³, whereas p50 processed in the absence of p65/I κ B translocates into the nucleus and functions as a homodimer, KBF1 (ref. 4). Thus, the cytoplasmic localization properties of the two NF- κ B subunits may have coevolved: one (p50) through linkage to ankyrin repeats in the C terminus of its precursor, and the other (p65) through association with a protein containing ankyrin repeats (I κ B; ref. 15). □

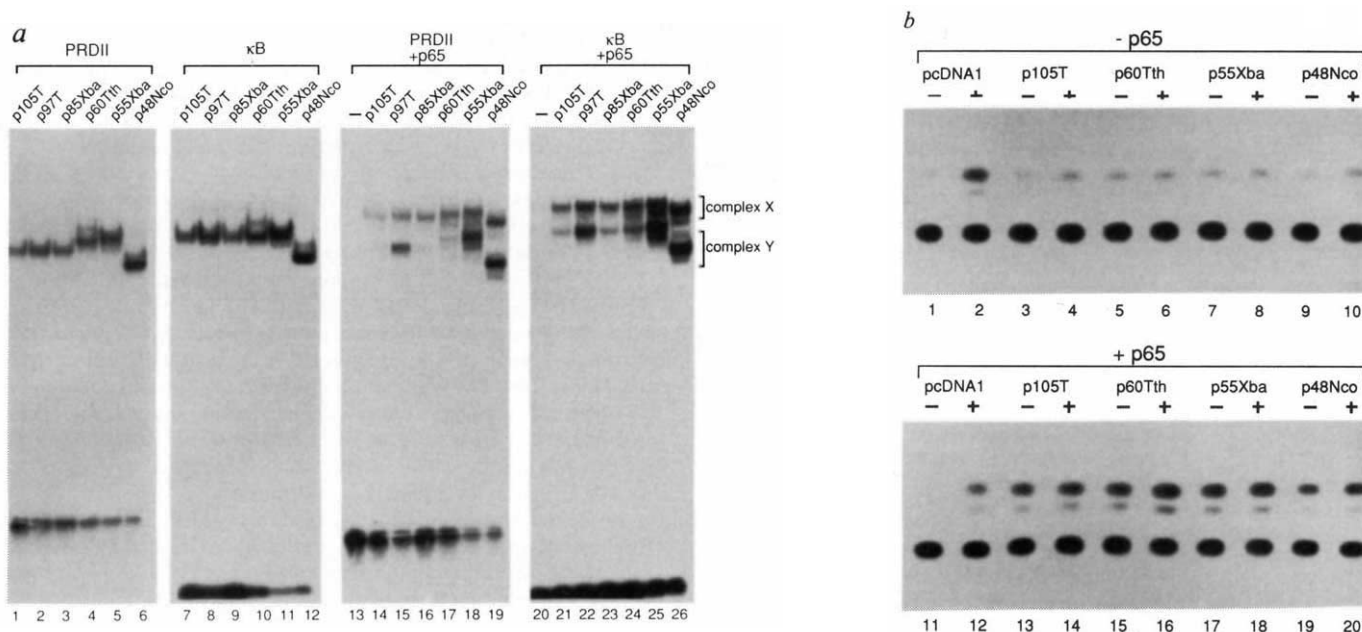


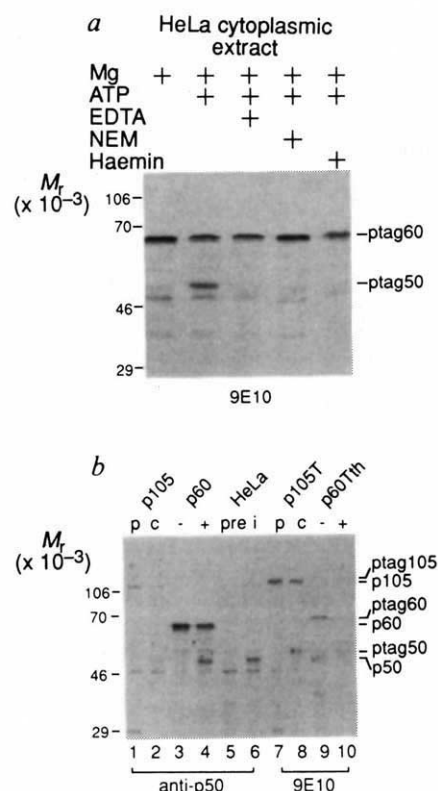
FIG. 3 **a**, Gel shift analysis of lysates from COS cells transfected with p105 deletion plasmids. The expression plasmids are indicated above each lane. In lanes 13 and 20, the minus sign indicates that only p65 was transfected into COS cells. DNA probes used for binding, PRDII and κ B (ref. 11), are indicated on top of each panel. The DNA-protein complexes of p50 variants are marked as complex Y, and the complexes of p65 and p50 variants, complex X. **b**, chloramphenicol acetyltransferase (CAT) assays of extracts from cells cotransfected with different combinations of expression vectors (as indicated) and the test plasmid -41 β (P)₂ (a human β -globin -41 promoter deletion linked to two copies of the PRDII site^{11,12}). - and +, Transfected cells mock-induced and virus-induced, respectively. p97T and p85Xba behaved similarly in the above assays (not shown). p105T, p60Tth, p55Xba and p48Nco failed to activate transcription through the PRDII (or κ B) site, and suppressed PRDII (or κ B) dependent virus-induced gene expression, presumably by dominant inhibition. Overexpression of NF- κ B p65 alone did not activate the basal level of transcription, and slightly

repressed virus-induced expression from the test plasmid (lanes 11 and 12). But cotransfection of p65 and p105 deletions activated transcription from promoters linked to PRDII or the κ B site but not from promoters without these sites (not shown).

METHODS. The NF- κ B p65 expression plasmid (p65) was made by cloning polymerase chain reaction-generated full-length mouse p65 cDNA⁶ into the *Bam*HI site of pcDNA1. For gel retardation assays, the probes containing PRDII or κ B sites were end-labelled using [α -³²P]dGTP, and the binding conditions and gel running buffer were as described¹¹. For transfection and CAT assays: 10 μ g of each expression vector (including p65 and control plasmid pcDNA1) and 2 μ g of test plasmid were used for transfection into mouse L929 cells. Transfection, virus induction and preparation of cell extracts were as described¹². All CAT assays were normalized to β -galactosidase activities produced from cotransfected control plasmid pRSV- β gal (2 μ g per transfection)¹².

FIG. 4 Generation of p50 from p60Tth precursor *in vitro*. **a**, *In vitro* processing of p60Tth with HeLa cell cytoplasmic extract. +, The components included in the reaction in addition to the basic buffer (see below). Positions of p60Tth (ptag60) and p50 (ptag50) are labelled. **b**, Comparison of the *in vivo* and *in vitro* processed p50. Lanes 1 and 2 are pulse-labelled (p) and chased (c) untagged p105 produced in COS cells; lanes 3 and 4, control-processed (-) and processed (+) untagged p60 (p60, translated *in vitro*) and p50 (p50) generated *in vitro*. Lanes 5 and 6 are *in vivo* labelled HeLa cell lysate. The above samples were immunoprecipitated with polyclonal antibodies indicated (pre, preimmune serum, and i, immunized serum to p50). Lanes 7 and 8 are pulse-labelled and chased p105T (ptag105) and tagged p50 (ptag50) produced in COS cells; lanes 9 and 10, control-processed and processed tagged p60Tth (ptag60) and p50 (ptag50) produced *in vitro*. The above samples were immunoprecipitated with 9E10.

METHODS. The p60Tth precursor was translated in wheat-germ extract (with ³⁵S-methionine) with the RNA transcribed by T7 polymerase from the p60Tth (Fig. 2a) plasmid (or an equivalent plasmid without Myc-tag). ATP and Mg²⁺ in the translation mix were removed by a G25 desalting column (Pharmacia) after translation reaction and RNaseA treatment. Protein (50 μ g) from HeLa cell cytoplasmic extract was used in each processing reaction. The basic processing buffer contained 12 mM Tris-HCl (pH 7.9), 60 mM KCl, 3.5 mM MgCl₂, 0.1 mM EDTA, 12% glycerol, with or without 20 mM creatine phosphate and 1 mM ATP. EDTA (5 mM), 2.5 mM NEM, and 100 μ M haemin were included as inhibitors in the indicated reactions. Control processing, same reaction without the addition of ATP and Mg²⁺. After incubation at 30 °C for 1 h, the reaction mixtures were subjected to immunoprecipitations with monoclonal 9E10 or polyclonal antibodies to p50, and different species of polypeptides were resolved by SDS-PAGE. COS cells were pulse-labelled (0.1 mCi of Tran³⁵S-label) for 20 min and chased for 60 min, and HeLa cells were labelled for 6 h (0.5 mCi) and chased for 1 h.



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Structural motifs and potential σ homologies in the large subunit of human general transcription factor TFIIE

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THE general transcription factor TFIIE has an essential role in eukaryotic transcription initiation together with RNA polymerase II and other general factors^{1,2}. Human TFIIE consists of two subunits of relative molecular mass 57,000 (TFIIE- α) and 34,000 (TFIIE- β)^{3–5} and joins the preinitiation complex after RNA polymerase II and TFIIF (ref. 5). Here we report the cloning and structure of a complementary DNA encoding a functional human TFIIE- α . TFIIE- α is necessary for transcription initiation together with TFIIE- β , and recombinant TFIIE- α can fully replace the natural subunit in an *in vitro* transcription assay. The sequence contains several interesting structural motifs⁶ (leucine repeat, zinc finger and helix–turn–helix) and sequence similarities to bacterial σ factors that suggest direct involvement in the regulation of transcription initiation.

Partial amino-acid sequences of human TFIIE- α was determined from five cyanogen bromide peptides (underlined regions in Fig. 1). With primers based on these sequences, polymerase chain reaction (PCR) and plaque hybridization techniques were used to isolate partial TFIIE- α cDNA clones from human placenta⁷ and Namalwa cell⁸ cDNA libraries. One (p2EYC) was found to contain a 1.7-kilobase (kb) insert with all of the peptide sequences derived from the purified TFIIE- α present in a long open reading frame (ORF). The putative ORF encodes a 439-amino-acid polypeptide with a calculated relative molecular mass (M_r) of 49,562 (Fig. 1).

Northern analysis with the TFIIE- α cDNA probe revealed a 3.2-kb transcript (data not shown), indicative of a unique TFIIE- α messenger RNA with long untranslated regions. Genomic Southern analysis showed single hybridizing DNA fragments (in *Bam*HI and *Pst*I digests), indicating that the human TFIIE- α is encoded by a single-copy gene (data not shown).

To demonstrate that the cloned cDNA encodes a functional TFIIE- α , the basal transcription activity of expressed proteins was tested with a reconstituted transcription system^{3,9}. A polypeptide of apparent M_r 57K, which shows the same mobility as purified TFIIE- α , was generated by *in vitro* translation of cDNA-derived transcripts in a reticulocyte lysate¹⁰ (Fig. 2a) or by expression of the cDNA in bacteria¹¹ (data not shown). In previous studies^{3,5} natural TFIIE- α displayed basal level transcription in a system containing partially purified human native TFIIB and TFIIG. But when assayed with the recombinant TFIIB (ref. 12) and HPLC-purified TFIIG (ref. 13), both the bacterially expressed and the natural α subunit showed transcription activity in the presence of the natural β subunit (Fig. 2b, lanes 4 and 6), whereas neither the α subunit (lanes 3 and 5) nor the β subunit (lane 7) alone showed any activity. These results demonstrate that the isolated clone encodes a functional TFIIE- α and that both α and β subunits are necessary for TFIIE function in transcription initiation.

As it is believed that TFIIE acts at a very late step in transcription initiation^{1,2,5}, it is instructive to consider sequences or motifs that might be involved in protein–protein or DNA–protein interactions. The deduced amino-acid sequence of TFIIE- α contains

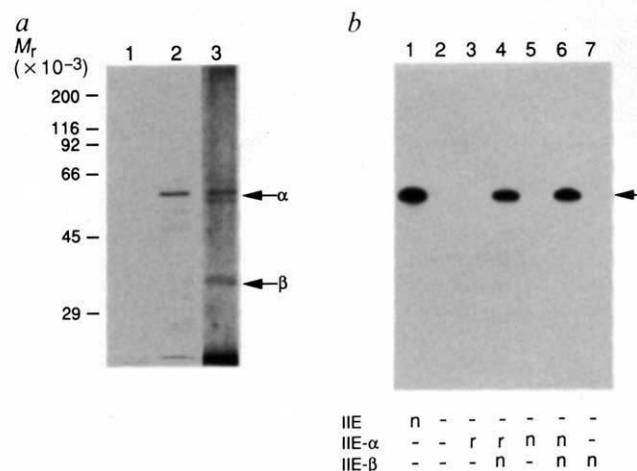


FIG. 2 Expression and function of cloned TFIIE- α . *a*, SDS-PAGE of [³⁵S]methionine-labelled TFIIE- α produced in a reticulocyte lysate. *In vitro* translation was done with no RNA (lane 1) or cloned TFIIE- α -derived mRNA (lane 2). Heparin-HPLC-purified TFIIE- α was run at the same time and silver-stained (lane 3). Arrows, positions of TFIIE- α and β . *b*, *In vitro* transcription by expressed recombinant TFIIE- α . Reactions containing the adenovirus major late promoter template, pML(CAT) Δ -50 (ref. 13) and general transcription factors TFIIB, TFIID, TFIIF, TFIIG and RNA polymerase II (refs 3, 13) were complemented with natural TFIIE (lane 1), buffer only (lane 2), bacterially expressed recombinant TFIIE- α (lane 3), recombinant α and renatured natural TFIIE- β (lane 4), renatured natural α (lane 5), renatured natural α and β (lane 6), and renatured natural β (lane 7). *r*, *n*, Recombinant and natural proteins, respectively. Arrow, position of the specific transcript. METHODS. A human TFIIE- α cDNA (nucleotides 1–1,599) in pBluescript SK(–) was transcribed with T7 RNA polymerase (Stratagene) and derived RNA was translated with rabbit reticulocyte lysate (Promega). The TFIIE- α expression vector was constructed as follows. An *Nde*I site (5'-CATATG) was made at the translation start site by site-directed mutagenesis of the p2EYC cDNA. The *Nde*I and *Bam*HI fragment was cloned into the pET3a plasmid¹¹. TFIIE- α was expressed in *E. coli* BL21(DE3)pLysS (ref. 11) by induction with IPTG (isopropylthiogalactoside). Lysates were prepared by sonication of cells in the buffer containing 20 mM Tris-HCl (pH 7.9), 500 mM NaCl, 20 mM 2-mercaptoethanol, 1 mM phenylmethylsulphonyl fluoride, 20 μ g ml^{–1} leupeptin, 20 μ g ml^{–1} pepstatin and 10% glycerol. TFIIE- α was partially purified through two columns (DEAE-cellulose and heparin-Sepharose). Transcription assay was performed as described³, except bacterially expressed human TFIIB (ref. 12) (20 ng) and TFIID (ref. 30) (30 ng), HPLC-purified TFIIG (ref. 13) and a saturating amount of recombinant TFIIE- α (1 ng) were used.

METHODS. Reversed-phase HPLC-purified TFII ϵ - α (ref. 3) (40 μ g) was digested with CNBr and some of the derived fragments (I–V) were sequenced. These sequences (with uncertain residues indicated by X or in parentheses) were: I, NH₂-RVETAADGKT(T/S)RHNYFYINRYTLNVVVKYKLD-COOH; II, NH₂-(R/X)RIETDERD(S/T)XNXASX(K)-COOH; III, NH₂-(L/E)-HRASLEGKSAKEPHIXLR (S/T)(V/Q)(G/A)(Y/G)-COOH; IV, NH₂-DAFQEREQEKAGPPDNEE-COOH; V, NH₂-RALLIHEKTS-COOH. For the first PCR, the sense and antisense primers based on fragment I/IV residues 1–6 and 13–18 were 5'-GA(C/T)GC(A/G/C/T)TT(C/T)CA(A/G/GA(A/G)(A/C)G-3' and 3'-GG(A/G/C/T)CT(A/G)TT(A/G)CT(C/T)CT(C/T)-5', respectively. A 54-bp PCR fragment was obtained. The internal antisense sequence (3'-CCAGCATGGCCTTCTC-5'), which corresponds to fragment IV residues 7–12, was used as a primer for the second PCR together with the sense primer 5'-GA(A/G)GGG-IAA(A/G)(A/T)(G/C)IGCIIAA(A/G)GA-3' based on fragment III residues 6–12. Inosine (I) was used instead of a four-nucleotide mixture. A 135-bp fragment was obtained by PCR amplification using a human Namalwa cDNA library⁸ (both oligo(dT) and random primed). This fragment was used to screen a human placenta cDNA library⁷ (oligo(dT)-primed). A partial cDNA (p2EYA) was obtained whose 5'-end (a 232-bp *EcoRI*-*NsiI* fragment) was used to isolate clones which contained the N-terminal region of TFII ϵ - α from the Namalwa cDNA library. One clone (p2EYC) was found to encode all of the peptide sequences. Screening was done under standard high-stringency conditions.²⁶

CTTGTATATTATTAAG	TGG	AGTT	CGT	TGCT	AAAG	ATG	GCA	GAC	CCA	GAT	GTC	CTC	ACT	GAA	GTT	64					
	M	A	D	P	D	V	I	R	G	F	Y	G	I	E	H	124					
CCA	GCA	GCA	TTG	AAG	CGG	TTA	GCC	AAG	TAT	GTG	ATC	CGG	GGA	TTT	TAT	GGC	ATT	GAG	CAT	124	
P	A	A	L	K	R	L	A	K	Y	V	I	R	G	F	Y	G	I	E	H	30	
GCC	TTG	GCC	TTG	GAC	ATC	TTG	ATC	AGG	AAC	TCC	TGT	GTG	AAA	GAG	GAG	GAT	ATG	CTG	GAG	184	
A	L	A	L	D	I	L	I	R	N	C	S	C	V	K	E	E	D	M	L	50	
CTG	CTC	AAG	TTT	GAT	CGG	AAG	CAA	CTT	CGA	TCA	GTT	TTG	AAT	AAT	TTA	AAG	GGA	GAG	AAG	244	
L	L	K	F	D	R	K	Q	L	R	S	V	L	N	N	L	K	G	D	K	70	
TTT	ATC	AAA	TGC	AGA	ATG	AGG	GTA	GAG	ACT	GCT	GCA	GAC	GGG	AAA	ACC	ACT	CGC	CAT	AAC	304	
F	I	K	C	R	M	R	V	E	T	A	A	D	G	K	T	T	R	H	N	90	
TAC	TAC	TTC	ATC	AAT	TAT	CGT	ACT	CTT	GTT	ATT	GTG	ATA	GAA	TAT	AAA	CTG	GAG	CAC	ATG	364	
Y	Y	F	I	N	Y	R	T	L	V	N	V	V	A	K	Y	K	L	D	H	M	110
AGA	AGA	AGA	ATT	GAG	ACC	GAT	GAG	AGA	GAT	TCG	ACC	AAC	CGG	GCT	TCC	TTC	AAA	TGT	CCT	424	
R	R	R	I	E	T	D	E	R	D	S	T	N	R	A	S	F	K	C	P	130	
II																					
GTG	TGT	AGT	AGT	ACT	TTT	ACA	GAT	TTA	GAA	GCT	AAT	CAG	CTC	TTT	GAT	CCT	ATG	ACA	GGA	484	
V	C	S	T	T	T	T	D	L	E	A	N	Q	L	F	D	P	M	T	G	150	
ACT	TTG	CGC	TGT	ACT	TTT	TGC	CAT	ACA	GAG	GTA	GAA	GAG	GAT	GAA	TCA	GCA	ATG	CCC	AAA	544	
T	F	R	C	T	F	C	H	T	E	V	E	E	D	E	S	A	M	P	K	170	
AAA	GAT	GCA	CGC	ACA	CTT	TTG	GCA	AGG	TTT	AAT	GAA	CAA	ATT	GAG	CCC	ATT	TAT	GCA	TTG	604	
K	D	A	R	T	L	L	A	R	R	F	N	E	Q	I	E	P	I	Y	A	L	190
CTT	CGG	GAC	ACA	GAG	GAT	GTG	AGC	TTG	GGC	TAT	GAA	ATA	CTT	GAG	CCA	GAA	CCC	ACA	GAA	664	
L	R	E	T	E	D	V	N	L	A	Y	E	I	L	E	P	E	P	T	E	210	
ATC	CCA	GCC	CTG	AAA	CAG	AGC	AAG	GAC	CAT	GCA	GCA	ACT	ACT	GCT	GGA	GCT	GCT	AGC	CTA	724	
I	P	A	L	K	Q	S	K	D	H	A	A	T	T	A	G	A	A	S	L	230	
GCA	GGT	GGG	CAC	CHC	CGG	GAA	GCA	TGG	GCC	ACC	AAA	GGT	GCT	TCC	TAT	GAA	GAC	TTA	TAC	784	
A	G	G	H	H	R	A	W	A	T	K	K	C	P	S	Y	E	D	L	Y	250	
ACT	CAG	AAT	GTT	GTG	ATT	AAC	ATG	GAT	GAC	CAA	GAA	GAT	CTT	CAT	CGA	GCC					

N-terminal regions with sequence similarity to bacterial σ factors¹⁴ (Fig. 3b), as well as three potential structural motifs⁶ (leucine repeat, zinc finger, and helix-turn-helix (HTH)) that are characteristic of many transcription factors (Fig. 3a). For each σ factor, the region with the greatest similarity to TFIIIE- α is the 2.1 region, which has been suggested to be involved in single-stranded DNA binding¹⁴. The basic and aromatic residues suggested to be important for this σ -factor function are well conserved (and even enriched) in TFIIIE- α ; TFIIIE- α contains a tyrosine at the position where a serine to tyrosine mutation enhances the activity of σ^{70} (residue 6 of the 2.1 domain)¹⁴. σ^{70} sequences N-terminal to and including the 2.1-2.2 region, as well as a homologous region in RAP30 (the small subunit of TFIIIF), have a bacterial core RNA polymerase-binding activity¹⁵. TFIIIE- α also has sequence similarity to these regions, consistent with the finding that partially purified TFIIIE can bind directly to RNA polymerase II (ref. 16). A region of

TFIIIE- α that overlaps both the σ 2.1-2.2 similarity region and part of the leucine repeat (residues 13-60) also has a high sequence similarity to the σ 4.1-4.2 region (Fig. 3c). Residues 33-60 have sequence similarity to the σ 4.2 region and contain a putative helix-turn-helix (HTH) structure. This σ 4.2 region (especially the HTH region) has been postulated to contact the -35 region of bacterial promoter sequences¹⁴. Although the extent of sequence similarity in this HTH region is not so high (especially in the second helix region, which is usually important for DNA-binding specificity), this HTH region might have a different DNA-binding activity from that of the σ 4.2 region. The overall σ -similarity region might have either DNA-binding activity (to either single-stranded or duplex DNA) or RNA polymerase-binding activity.

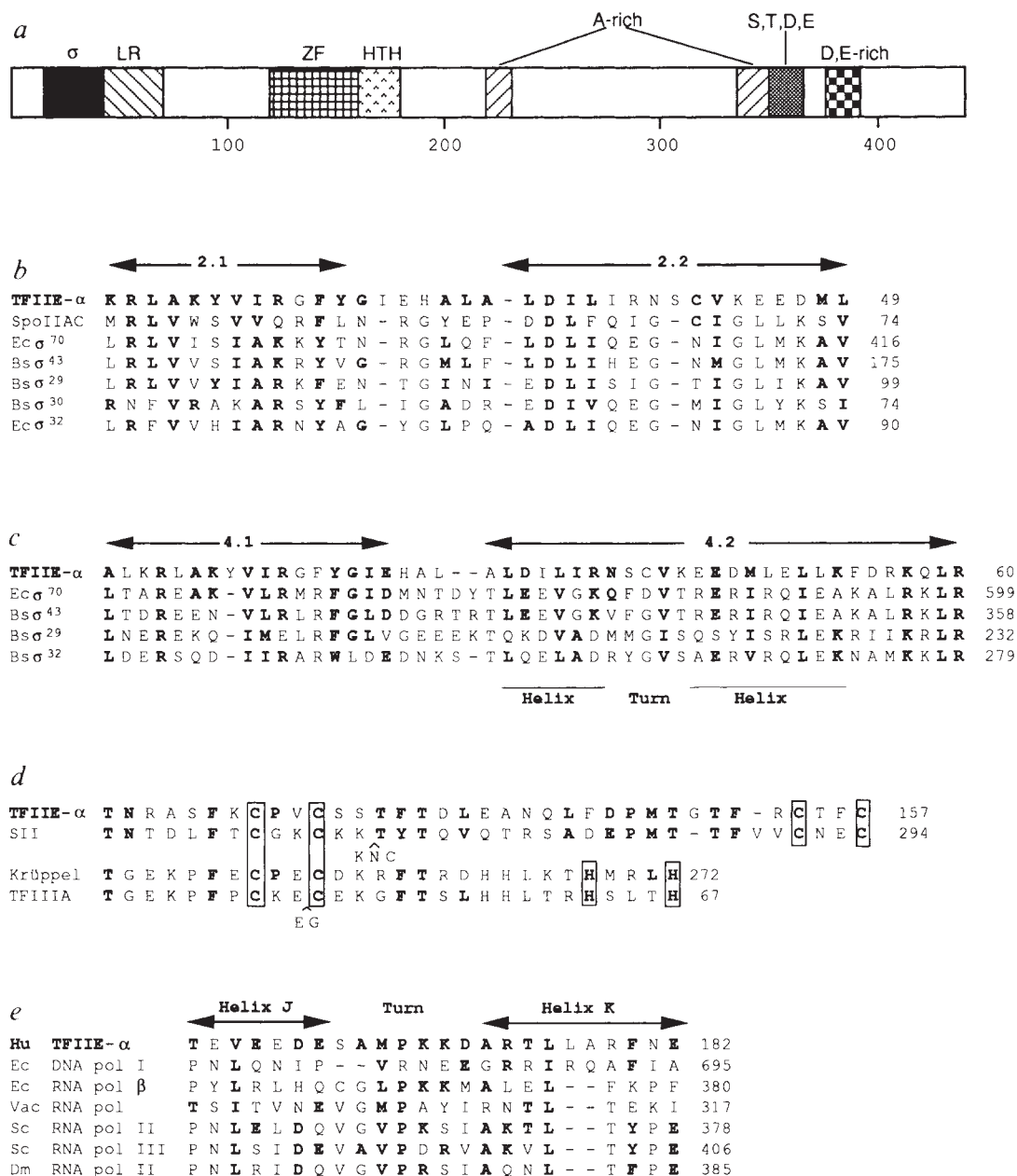
C-terminal to the putative σ homology there is a putative amphipathic α helix with a leucine repeat (residues 38-66) that could be involved in homomeric and/or heteromeric interactions

FIG. 3 Structural features

of TFIIIE- α . a, Schematic diagram of representative structures found in TFIIIE- α . σ , Similarity to bacterial σ factors; LR, leucine repeat; ZF, zinc-finger; HTH, helix-turn-helix; A-rich, alanine-rich; S, T, D, E, region consists of serine, threonine, aspartic acid and glutamic acid; D, E-rich, acidic region.

b, Alignment with bacterial σ -factor regions 2.1-2.2. *E. coli* (Ec) and *Bacillus subtilis* (Bs) σ -factor sequences are from ref. 13. SpoIIAC is from a *B. subtilis* phage. The position in the protein sequence of the last residue in each line is indicated in the last column. Amino acids identical (or conserved) to those in TFIIIE- α are in bold according to the grouping (E, D), (K, R, H), (N, Q), (G, P), (A, I, L, V, M), (F, W, Y), (C) and (S, T). The most highly conserved domain has similarity to the σ -factor 2.1 region and the first half of the 2.2 region.

c, Alignment with bacterial σ -factor regions 4.1-4.2. The region which completely overlaps with the above mentioned σ 2.1-2.2 region and partially overlaps with the leucine-repeat region also has good similarity to the σ -factor regions 4.1-4.2. The HTH region is underlined. d, Zinc-finger. Transcription elongation factor S-II (ref. 19) has the strongest similarity to TFIIIE- α and both polypeptides possess the C₂C₂ motif (boxed). TFIIIE- α also contains well conserved residues found in the 5S gene-specific TFIIIA and the *Drosophila* developmental protein Krüppel (boxed C₂H₂ motifs). e, HTH. Helix J and helix K are helices found initially in *E. coli* DNA polymerase I. Hu, human; Ec, *E. coli*; Vac, vaccinia virus; Sc, *Saccharomyces cerevisiae*; Dm, *Drosophila melanogaster*.



with other leucine repeat-containing transcription factors⁶ (Fig. 3a)¹³. The second motif is a putative zinc-finger containing several residues that are conserved in the C₂H₂ (two cysteine and two histidine) zinc-finger structures present in the 5S gene-specific TFIIB (ref. 17) and the *Drosophila* Krüppel protein family¹⁸ (Fig. 3d). But unlike these proteins, which contain multiple fingers of the C₂H₂ class, TFIIE- α contains a single putative zinc-finger of the C₂C₂ (two cysteine pairs) class. Thus the structure and function of the putative TFIIE- α zinc-finger might be more similar to that of the single zinc-finger in transcription elongation factor S-II (ref. 19), suggesting an involvement either in DNA-strand opening or protein-protein interactions. The third motif is a HTH with sequence similarity (and probable homology) to the largest subunit of both prokaryotic and eukaryotic RNA polymerases and *Escherichia coli* DNA polymerase I (refs 20, 21) (Fig. 3e). This region has been proposed as a DNA-binding region which interacts through the major groove of duplex DNA^{20,22}, although there is little similarity to the 'classical' HTH motif which is contained, for instance, in homeodomain proteins.

All four of these conspicuous motifs reside in the N-terminal 182 residues of TFIIE- α and can be grouped in two domains. The first domain (residues 13–66), consisting of the 'σ' and 'leucine repeat' regions (Fig. 3a, b, c), is quite basic (pI 9.5). This domain might form a novel basic region/leucine zipper structure, with the σ region replacing the usual basic region, which might recognize (perhaps alternately) either duplex or single-stranded DNA. The second domain (residues 121–182), containing the adjacent 'zinc-finger' and 'HTH' motifs (Fig. 3a, d, e), could bind to double-stranded DNA. Both domains might be important for transcription initiation by aiding the DNA-melting reaction. In contrast, no known motifs were identified in the C-terminal half of TFIIE- α , which might therefore contain novel functional interaction domains.

Note that TFIIE- α (mainly the C-terminal 300 residues) is highly acidic (pI 4.5) with several acidic clusters²³. By contrast, TFIIE- β , the smaller subunit of TFIIE, is highly basic (pI 9.5)²⁴, suggesting that the two subunits could easily complex by ionic interactions. TFIIE- α may also interact with other positively charged transcription factors such as initiation factors TFIID (refs 25, 26) and TFIIB (ref. 27).

Sequence similarities of TFIIE to structural motifs found in the essential regions of RNA polymerases^{2,28} and other transcription factors, regions of similarity with bacterial σ factors, and the presence of acidic clusters suggest various potential interactions with other transcription factors. With the above structural motifs in mind, it will be intriguing to examine how TFIIE functions, through protein-protein and DNA-protein interactions, during preinitiation complex formation and activation (including transitions from a closed to an open complex and from initiation to elongation). □

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Conserved sequence motifs in the small subunit of human general transcription factor TFIIE

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A GENERAL initiation factor, TFIIE, is essential for transcription initiation by RNA polymerase II in conjunction with other general factors^{1,2}. TFIIE is a heterotetramer containing two subunits of relative molecular mass 57,000 (TFIIE- α) and two of 34,000 (TFIIE- β)^{3,4}. TFIIE- β is required in conjunction with TFIIE- α for transcription initiation. Here we report the cloning and expression of a complementary DNA encoding a functional human TFIIE- β . Recombinant TFIIE- β could replace the natural TFIIE- β for transcription in conjunction with TFIIE- α . Amino-acid sequence comparisons reveal regions with sequence similarities to: subregion 3 of bacterial σ factors⁶; a region of RAP30 (the small subunit of TFIIF) with sequence similarity to a σ-factor subregion implicated in binding to RNA polymerase⁷; and a portion of the basic region-helix-loop-helix motif found in several enhancer-binding proteins^{8–10}. These potential homologies have implications for the role of TFIIE in preinitiation complex assembly and function.

Four cyanogen bromide (CNBr)-digested peptides (I–IV) of TFIIE- β purified from HeLa cells⁴ were separated by reversed-phase HPLC and their amino-acid sequences determined. With primers based on these sequences, amplification of a human cDNA fragment by polymerase chain reaction (PCR) yielded a 289-base-pair fragment containing the expected sequences. This fragment was used to screen a human cDNA library derived from Namalwa cells¹¹ and several overlapping clones were isolated and sequenced. Sequences encoding the four CNBr-derived peptides were found in an open reading frame of 873 nucleotides which encodes a polypeptide of 291 amino acids with a calculated relative molecular mass of 33,040 (Fig. 1). An RNA blot indicated that the human TFIIE- β messenger RNA is around 1.6 kilobases (kb), close to the length of the cDNA, and no difference in size was found between the Namalwa and HeLa cell mRNAs (H.S. and E.S., unpublished results). Only one major cross-hybridizing restriction fragment was detected in human genomic DNA digested with *Hind*III (H.S. and E.S., unpublished results), suggesting that there is one copy of the TFIIE- β gene per genome.

To assess the function of the cDNA-encoded protein, it

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The predicted amino-acid sequence of TFIIE- β is not closely related to any other proteins in the GENBANK and EMBL databases. But direct comparison of the TFIIE- β amino-acid sequence with sequences present in bacterial σ factors⁷ indicated some similarities. The highest similarity is in the σ -factor region 3 (Fig. 3b). Although this region has been suggested to be

METHODS. Reversed-phase HPLC-purified TFIIIE- β (ref. 4) (25 μ g) was digested by CNBr and the resulting polypeptides (I–IV) were separated and sequenced. These sequences in the single-letter amino-acid code, with an uncertain residue indicated by X, where: I, NH₂-DPSLLRERLFKKRAI-STPVVEKRA-COOH; II, NH₂-KTRHQRGDLTHPLT-DEILDETQHLDI-COOH; III, NH₂-DEEKIEELKRGQIS-COOH; IV, NH₂-QESGPKKVAPIQRKKPKASQKKR-RFKCOOH. For PCR, degenerate sense and antisense primers were synthesized on the basis of the amino-acid sequence of fragments I and II, with additional 5' extensions carrying *Sal*I and *Xba*I sites, respectively: 5'-GCGTCGACICCTGTG-TTGA(A/G)AA(A/G)-3' and 3'-CT(AG)CT(T/C)TG-TGT(T/C)GT(A/G)(A/G)AICTAGATCAG-5'. Inosine (I) was used instead of a four nucleotide mixture in degenerate positions. A 289-bp fragment was isolated by PCR amplification using a λ ZAP phage library derived from Namalwa cells¹¹. Sequencing of the subcloned fragment revealed that it encodes polypeptide-containing amino-acid sequences of CNBr-derived peptides I and II. The *Sal*I–*Xba*I fragment of the clone was used as a probe to screen 1.2×10^6 recombinants from the same library. Fourteen independent clones were sequenced in both directions using the dideoxynucleotide chain termination method.

A region of TFIIE- β also shows sequence similarity with a region of RAP30 (Fig. 3c). The latter is part of a larger RAP30 domain with sequence similarity to a σ^{70} domain (including subregions 1b, 2.1 and 2.2), which binds to core RNA polymerase⁶. Given that both TFIIE and TFIIF bind independently to RNA polymerase II (ref. 19), these interactions could involve, at least in part, the related domains indicated in Fig. 3c. Therefore, these results provide one of the first examples of a similarity between the eukaryotic general initiation factors. This finding further supports the suggestion that TFIIE- β and RAP30 may be derived from a common ancestor (σ factor). Although TFIIE- α also has similarity to the same region of RAP30 and σ^{70} (ref.

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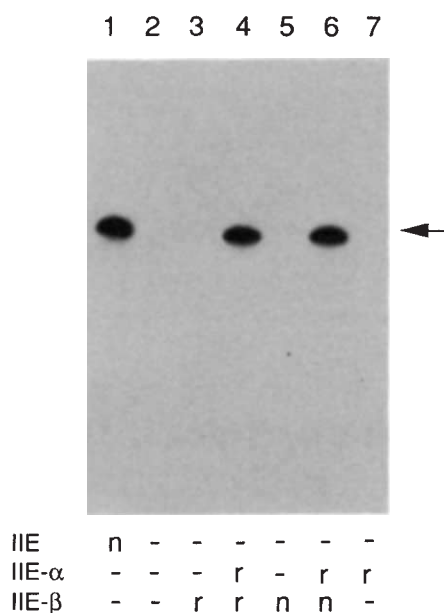
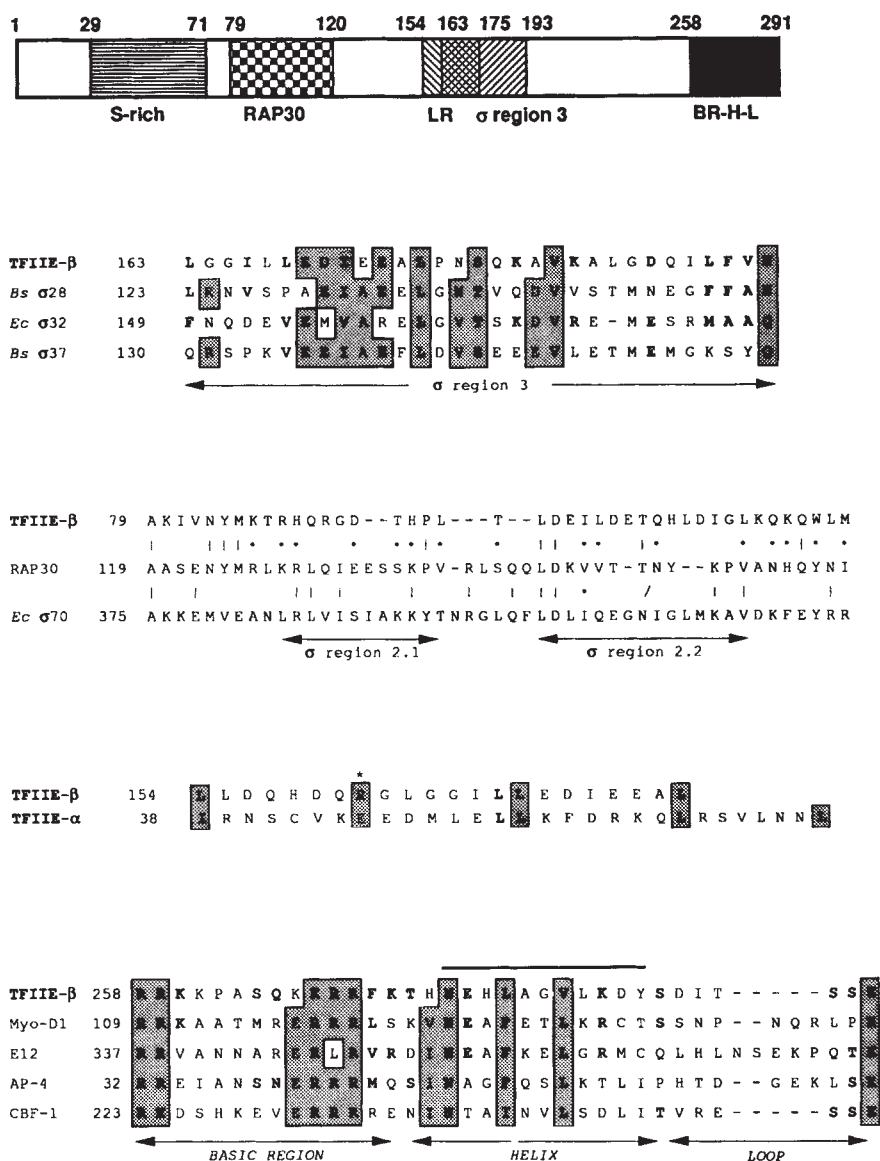


FIG. 2 Transcription activity of expressed human TFIIIE-β in a reconstitution system. Arrow indicates position of the specific transcript from a template of the adenovirus major late promoter, pML(C₂AT)Δ-50 (ref. 15). Reaction mixtures containing pML(C₂AT)Δ-50, RNA polymerase II, TFIIIB, TFIIID, TFIIIF and TFIIIG (refs 4, 16) were complemented with natural TFIIIE (lane 1), buffer only (lane 2), bacterially expressed recombinant TFIIIE-β (lane 3), bacterially expressed recombinant TFIIIE-α and β (lane 4), renatured natural β (lane 5), recombinant α and renatured natural β (lane 6), and recombinant α (lane 7). n, r, Recombinant and natural proteins, respectively.

METHODS. The coding region of a human TFIIIE-β cDNA was cloned into *Nde*I and *Bam*HI sites of the pET3a plasmid¹² and expressed in *E. coli* by induction with IPTG. Lysates were prepared by sonication of the cells in buffer A (20 mM HEPES, pH 7.9, 500 mM NaCl, 10% glycerol, 1 mM EDTA, 10 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride, 5 μg ml⁻¹ leupeptin, 1% aprotinin and 0.1% nonidet P-40). The expressed TFIIIE-β was purified from the lysates by (NH₄)₂SO₄-fractionation: after addition of (NH₄)₂SO₄ to 33% saturation, the suspension was centrifuged at 12,000g for 45 min, and the precipitates were resuspended in buffer A. The bacterially expressed recombinant TFIIIE-α was prepared and transcription assays were performed as described⁵ except a saturated amount of recombinant TFIIIE-β (0.5 ng) was also used.

FIG. 3 Structural features of human TFIIIE-β. *a*, Schematic representation of motifs found in TFIIIE-β. S-rich, serine-rich region; RAP30, region similar to RAP30; LR, leucine repeat; σ region 3, region similar to bacterial σ-factor region 3; BR-H-L, basic region-helix-loop domain. Some part (residues 163-175) of LR overlaps with σ region 3 as indicated. *b*, Alignment of TFIIIE-β sequence with bacterial σ-factor sequences. *E. coli* (Ec) and *Bacillus subtilis* (Bs) σ-factor sequences are from ref. 7. The position in the first residue of the protein sequence in each line is indicated in the second column. Bold letters represent amino acids identical or similar to those in TFIIIE-β according to the grouping: (D, E), (N, Q), (K, R, H), (A, I, L, M, V, F), (S, T) and (Y, W). Amino acids that are relatively conserved among bacterial σ factors, proposed by Helmann and Chamberlin⁷, are shaded. *c*, Alignment of TFIIIE-β sequence with RAP30 sequence. RAP30 and *E. coli* (Ec) σ⁷⁰ sequences are from ref. 6. The sequence are aligned to give the best match between TFIIIE-β and RAP30, but a better match between TFIIIE-β and the σ-factor 2.1 subregion can be obtained by aligning TFIIIE-β residues 80-91 with σ⁷⁰ residues 385-396. *d*, Leucine repeat. The putative leucine repeat of TFIIIE-β is aligned with that of TFIIIE-α. Relevant residues of the seven-amino-acid repeat are boxed. Oppositely charged residues are at the same position (*) on top and will possibly interact ionically with each other on the same surface as the other repeated leucines. *e*, Basic region-helix-loop (BR-H-L) motif of TFIIIE-β. Amino-acid sequences are from MyoD1 (ref. 28), E12 (ref. 8), human AP-4 (ref. 29) and *Saccharomyces cerevisiae* CBF-1 (ref. 30). Amino acids conserved among the BR-HLH proteins, proposed by Lüscher and Eisenman⁹, are shaded. The horizontal bar on the upper side indicates the region of an amphipathic helix.



5), no conspicuous similarity can be found between α and β subunits. Moreover, good similarity to the σ region 2.2 can also be found overlapping with the σ region 3 similarity domain (residues 168–182). It is intriguing that a putative promoter recognition region and an RNA polymerase II binding domain are located together.

TFIIE- β is a highly basic protein of pI 9.5 with several basic amino-acid clusters through which it could easily complex with TFIIE- α , a very acidic protein with several acidic clusters⁵. One of the basic clusters in TFIIE- β lies in the serine-rich region (Fig. 3a) where several putative phosphorylation sites exist, suggesting that complex formation might be regulated by protein kinases. Another potential protein interaction site in TFIIE- β is a putative leucine repeat²⁰ from residues 154 to 176, of interest as TFIIE- α also has a potential leucine repeat⁵ (Fig. 3d). Two subunits in the heterodimer may interact through this domain.

Previous studies suggested that partially purified TFIIE fractions have an ATPase activity that might be involved in an ATP hydrolysis event needed for transcription initiation^{21,22}. But recent analyses failed to detect such an activity in a homogeneous preparation of natural TFIIE^{3,4,23}. Consistent with this observation, neither TFIIE- α (ref. 5) nor TFIIE- β (this study) has any of the characteristic sequence motifs found in ATP-binding proteins (including ATPases and helicases)²⁴.

Surprisingly, we also found a region of TFIIE- β with sequence similarity to part of the basic region-helix-loop-helix (BR-HLH) domain of the c-Myc-related family of enhancer binding proteins (such as Myo-D1 and E12)^{7–9}. In addition to the similarity in primary structure (Fig. 3e), the putative secondary structure of TFIIE- β seems to be consistent with such a motif, whereas the composition of residues 258–273 of TFIIE- β resembles basic regions of the type found in enhancer-binding proteins. Further, the Chou-Fasman algorithm predicts that residues 274–284 (the horizontal bar in Fig. 3d) have a high probability of forming an amphipathic helix. Consistent with the possibility of a loop structure^{7,25}, residues 285–291 have the potential to form a β turn and contain loop-favouring residues such as serine and aspartate. The BR-HLH domain may also be functionally bipartite, with the HLH segment being involved in homo- and heterotypic protein-protein interactions and the basic region in DNA binding^{7–9}. Hence the presence of related structures in TFIIE- β raises the possibility that it might be a DNA-binding protein with limited sequence specificity, possibly recognizing the DNA in conjunction with other factors, or that it might interact directly with enhancer bound BR-HLH proteins and thus have a role in transducing their effects to the basic transcription machinery.

Analysis of the protein sequence of TFIIB revealed structural features and potential homologies both to general and to gene-specific transcription factors from prokaryotes and eukaryotes. It should now be possible to analyse these features in relation to the role of TFIIE- β in preinitiation complex assembly, function and regulation. □

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Requirement of yeast fimbrin for actin organization and morphogenesis *in vivo*

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THE *SAC6* gene was found by suppression of a yeast actin mutation¹. Its protein product, Sac6p (previously referred to as ABP67), was independently isolated by actin-filament affinity chromatography and colocalizes with actin *in vivo*². Thus Sac6p binds to actin *in vitro*, and functionally associates with actin structures involved in the development and maintenance of cell polarity *in vivo*^{2,3}. We report here that Sac6p is an actin-filament bundling protein 43% identical in amino-acid sequence to the vertebrate bundling protein fimbrin⁴. This yeast fimbrin homologue contains two putative actin-binding regions⁵ homologous to domains of dystrophin, β -spectrin, filamin, actin-gelation protein and α -actinin. Mutants lacking Sac6p do not form normal actin structures and are defective in morphogenesis. These findings demonstrate an *in vivo* role for the well-documented biochemical interaction between fimbrin and actin.

To elucidate the molecular nature of the interaction between Sac6p and actin, we looked at the effect of Sac6p on assembly and organization of actin filaments *in vitro*. Thus, Sac6p was mixed with yeast actin under assembly-inducing conditions, and the resulting products were examined by electron microscopy. When pure yeast actin was assembled, a meshwork of actin filaments, with occasional aggregates, was observed (Fig. 1a). But when Sac6p was added at a molar ratio of one Sac6p per 20 or 10 actins, single filaments were not observed, all the actin appearing in filament bundles (Fig. 1b). This result was confirmed by anti-actin immunofluorescence microscopy (not shown), and indicates that Sac6p can bundle actin filaments.

SAC6 was sequenced to determine whether it is related to any of the known actin-filament bundling proteins (Fig. 2). A single long open reading frame was identified that encodes a protein of 642 amino acids, with a predicted relative molecular mass of 71,700. A search of the protein sequence database indicated that Sac6p is 43% identical to the chicken actin-filament bundling protein fimbrin⁴, and 44 and 38% identical, respectively, to human T- and L-plastin^{6,7}, previously shown to be human homologues of chicken fimbrin⁴. Sequence alignments for chicken fimbrin, yeast Sac6p and human L-plastin (Fig. 3a) show that the homologies are extensive. Like fimbrin⁴, Sac6p is composed of two repeated domains of 254 and 257 amino acids

that are 26% identical to each other, preceded by a unique region (131 amino acids for Sac6p) at the amino terminus. In each repeated domain lies a region of 27 amino acids that is very similar to putative actin-binding regions⁵ identified in ABP-120 (actin-gelation protein)⁸, fimbrin⁴, β -spectrin⁹, ABP-280 (filamin)¹⁰, dystrophin¹¹ and α -actinin¹² (Fig. 3b). In the amino terminus, as in fimbrin, there are two potential calcium-coordinating EF hand sequences¹³ (Fig. 3c). But in contrast to fimbrin, the positions of calcium-coordinating residues do not fit the consensus well enough to make it likely that these regions of Sac6p bind to calcium ions¹³.

To test the function of Sac6p *in vivo*, a complete *SAC6* gene deletion was constructed (see legend to Fig. 4). The null mutant is viable, but grows at a reduced rate at 23 °C (150–215 min doubling time for the mutant compared with 115 min for wild type; the ranges in growth rate are due to variations seen in three different mutant strains with related, but nonidentical genetic backgrounds, described in the legend to Fig. 4). The null mutant fails to grow at 37 °C, undergoing less than a 1.4-fold increase in cell number after the shift. At 37 °C, the mutant cells show a slight increase in the proportion of unbudded cells, but arrest at all stages of the cell cycle. In addition, these cells are somewhat more heterogeneous in size than at 23 °C, and many of them appear lysed. These results indicate that Sac6p is advantageous to cells at lower temperatures and has an essential function at high temperature, and suggest there may be some other protein(s) that can functionally substitute for *SAC6* at the lower temperatures. Similar suggestions of partial functional redundancy in the actin cytoskeleton have been made for *Dictyostelium*¹⁴.

By immunofluorescence microscopy, the *sac6* null mutant has an altered actin cytoskeleton, even at 23 °C. As a temperature shift affects even the wild-type actin distribution (our unpublished observations), we have characterized the phenotype at 23 °C, using strains AAY1040, AAY1041, and AAY1042 (*sac6::LEU2/sac6::LEU2*) and AAY1043 (*SAC6⁺/SAC6⁺*) (see legend to Fig. 4). First, we have found that whereas extensive actin cables are seen in 90% of wild-type cells (Fig. 4b), cables are visible in only 22% of the mutant cells (strain AAY1041), and those cables that are seen are drastically reduced in intensity, frequency and length (Fig. 4d, e; note that the wild-type actin cables visible in the microscope are very under-represented in Fig. 4b, as most actin cables are in a different focal plane from the more cortically located actin patches). This observation supports a role for Sac6p in cross-linking actin filaments *in vivo*. Second, the cortical actin patches normally concentrated at the growing surfaces of the bud (Fig. 4b)^{15,16} show this asymmetry in only 3% of the mutant budded cells (strain AAY1041). Instead, the actin patches are fairly uniformly distributed at the cortex (Fig. 4d) of 79% of the mutant cells and are aberrantly clustered (Fig. 4e) in the remaining 18% of the cells. In this latter class, the clusters are aberrant in that they cover an abnormally large surface area and, in contrast to wild type, do not occur exclusively in the bud (compare Fig. 4b, cells a–d with the corresponding cells a'–d' in e). The actin distribution described for AAY1041 is similar to that seen in AAY1040 and AAY1042, although there is some variability in relative frequencies; however, even in AAY1042, the strain most different from AAY1041, 56% of the cells have no detectable actin cables, and 79% show the abnormal distributions of actin patches shown in Fig. 4d, e.

Interestingly, the mutant cells are much rounder than wildtype (Fig. 4a, c). As this phenotype is reversed by *SAC6* on a low-copy-number plasmid (not shown), the defect is caused by the *sac6* null mutation. This observation supports the notion that the polarized distribution of actin seen in normal cells is important in the polarization of growth¹⁵.

Fimbrin had previously been found in vertebrate cells and shown to bundle actin filaments *in vitro*^{17–20}. Our findings suggest that fimbrin homologues are likely to be found in association

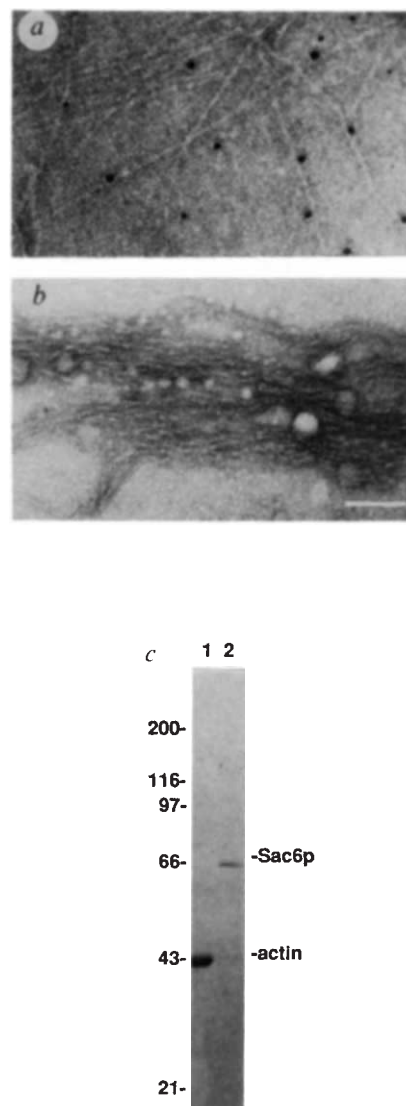


FIG. 1 Electron micrograph of negatively stained yeast actin filaments (a) and yeast actin filament bundles formed by the addition of Sac6p at a molar ratio of one Sac6p per 10 actins (b). All fields (8/8) containing Sac6p lacked individual (non-bundled) filaments, whereas all fields (5/5) lacking Sac6p contained individual filaments. Scale bar, 0.1 μ m. c, Coomassie blue stained SDS-polyacrylamide gel showing purified yeast actin (lane 1) and Sac6p (lane 2). In some Sac6p preparations, a small amount of yeast actin from the yeast actin column (see below) copurifies.

METHODS. Yeast actin was isolated by DNase1 affinity chromatography¹⁶ and, after desalting on a Sephadex G25 column (Pharmacia), was further purified using a Mono Q (Pharmacia) FPLC column. The actin eluted at roughly 0.2 M KCl using a 0 to 0.4 M KCl gradient (complete procedure to be described in detail by S. Kron, D.G.D., D.B. and J. Spudich, manuscript in preparation). Sac6p was isolated from a wild-type or *abp1* null mutant²² by actin-filament affinity chromatography as described by Drubin *et al.*². Sac6p was sometimes concentrated in a dialysis bag placed in Ficoll (*M*, 400K) and was dialysed into 5 mM HEPES, pH 7.4, 1 mM EDTA, 20 mM KCl, 10% glycerol, 0.5 mM DTT, 1 mM ATP and a 1X protease inhibitor cocktail². To determine effects of Sac6p on actin filament assembly and organization, Sac6p (or dialysis buffer alone for control experiments) was brought to 5 mM $MgCl_2$ and 10 X concentrated monomeric yeast actin (30 μ M, stored in 5 mM Tris, pH 7.5, 0.2 mM ATP, 0.2 mM DTT and 50 μ M $MgCl_2$) was added to a final concentration of 3 μ M. After 90 min at 23 °C, samples were applied to ionized carbon stabilized formvar grids and negatively stained with 1% aqueous uranyl acetate for viewing by electron microscopy.

with actin in all eukaryotes; thus this association arose early in the evolution of eukaryotes. Our results also suggest that there is a functional association of these fimbrin homologues with actin in all cells, not just the highly specialized microvillar actin bundles where fimbrin was first discovered and characterized.

Our experiments also give us insight into the function in the living cell of the fimbrin-actin interaction. The failure to

properly organize actin results in a defect in morphogenesis, particularly as manifested by cell shape, even at the nominally permissive temperature of 23 °C. But the fact that the cells are able to grow at this temperature suggests that proper actin organization might be most important under stressful conditions, such as extreme temperature. We speculate that individual actin filaments can direct transport of materials to regions of growth

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1  GAGCTCAAGGACGCCCATTTGAGAATGACCTTCCTCGAGCTAAATTGAAAGGGAAGAATTTATTAGTTGAACCTAAGAAAGAAGAGGACGTGGGAAATGGCATAAGAATCCCTTACT
121 AAATCGAAGACTAACTGAATTCATGCTGGCGAACGAAGTAAGATACACAAAGCTAGTTTCCAGAAAAGTGTAAATTTAACTACCTGATAATATAGTACTGAAGAAACCGTGGAA
241 CTTAAAGAAATAAAGGACTTGCTACTACAAATGTTGAGAAGACAGCGAGAGATTGAATCAAGATTATCCAATATCGAACTTCAACTCACGGAATACCGAAACATAAGTAATCATATCCC
361 TTCTCACATTTTTTACACAGGAAGTAAGCAAGTTATGTTATATTTCCGACACTATAATTAATTCCTAGCAGTTAAAGGTGCTTTGTCTATATTACATTACATACAGCTTGAGTGATCCT
481 GACCGGATATAGGGTCTATTTTCTTACGTGAACGGCTTTTCTTCTGTTCCCGATGGCTTCATGTGAAAAGCACTCCTCGGGAGGCGGAAAAATATCAAAGTACGGGGCGAAGTTT
601 ATAATGAAGATTATTCGATATAAATTTTGGTTATTTTCAGGAGACAAGAAAGCTCTTTACACTAAAATATCAGAGAAGAAGCTGATATATTAGCCCTAAGGAGTACACCAAAACACAAT
601
721 GAATATTGTCAAATACAAATATGCTATGCCTTTCTCGAATCCACACTGCTGAGCACAGTGAAAAAGTTTTTCTACCTTTTAGGTTGTAATAAACGTTTCATATACATAACAACTCTT
721
841 CTGCGATTAGAGAAAAATTTCAATTTTGACTCAAGAGGATCTTTTTCCACAATGAAAAGTTCAGAGCCATTGACTTAGATGATAAAGGATGGTTGAAAAACAGCAAGCTCTAGAAGC
841
961 GGTGTCCAAAGATGGTGATGCTACTTATGACGAAGCAAGAAACCCCTGAAGCATGTGGGTGTGGATCGCTCAGGCGGTGTTGAAGTGGACGATTACGTGGGTGGTTGCAAAATTAAG
961
1081 AGAATCTAAAACAGGAGCTGCTCCACAGACCACTTTCAACGTAGCACCTAACTCAACCCCTATTGTTTCCACTGCTGCCACAGGACTACAGCAAGGTAAGGTTACTCAAGCCAAAGAT
1081
1201 TATTGTTGCTGGCTCCCAACAGGTACTACATACCATCAATGAAGAAGAAAGACGTGAATTTACTAAACATATCAATAGCGTCTTGGCGGGAGACCAAGATATAGGTGATTGCTTCC
1201
1321 ATTTCTACTGATACTTTCAATTTATTTGATGAATGATAGGATGGGTGGTGTCTATTAAGTTGATCAACGACTCTGTTCTGATACATATCGATACAAGAGTTTGAATTTGGCTAAGAA
1321
1441 GGGTAAGGAATTAATAATTTTCAAGCTAGTGAAATGCTAATATGTTTATTAATTCGAAAAGCCATTGGTTGTGCTGTTGTAATGTTTATCCGAGGACATTATGAAGGTAGAGA
1441
1561 GCATTGTGCTGGTGGTTGATTGGCAAAATTTAGGAGAGGTTTGTAAAGTAAATTTGATATTAAGCTGCATCCTGAGTTATACCGTCTATTGGAGGATGAGAAACGTTGGAGCAATT
1561
1681 TTTGAGGTTGCCTCCGAGCAAAATCTACTACGTTGGTTTAACTACCATTGAAGCAAGCAAACTGGAACAGAAGGTTACCAATTTTCAAAGACGTGTCTGATGGTGAAGCAATATA
1681
1801 TATCTGTTGAACAGTTGGACCCCGCTTGTGTTCTAAGGCACATTACAACACAGATTTAATGGAGAGAGCTGAGCAAGTTTACAAAATGCTGAAAGCTAGATTGTAGAAGTA
1801
1921 TTTAACCCTAAGTTTCAATAGTTGCGGGGAACCCCTAAATTAACCTGGCTTTCGTGGCACATCTATTCAATACACATCCTGGATTAGAGCCTATTGAGGAAGGAAAAGCCTGAAATTGA
1921
2041 AGAATTTGACGCCGAAGGTGAGAGAGAGGCAAGAGTCTTACCTTTGATGGTTGAACCTCTTGTGACGTTGATCCACCTGTCTTTCGCTGTTTGTGATTTGAAGGATGGTTGATATATT
2041
2161 GCAAGCTTACGAAAAGGTGATGCCCGGTGCTGTTGATTTTAAACATGTCAACAAAAGACCGGCTCGGGTGCAGAGATATCCAGATTCAAAGCCTTAGAAAACACCAACTATGCAGTCGA
2161
2281 CTTGGGCAGAGCCAAAGGATTTCTTGTGTTGATCGAAGGATCTGATATGTTAGATGGTAACAAATTAAGTACCTTAGGTTAGTGTGGCAATGTGCGTAGAATAATTTTCGATAAT
2281
2401 CATGAGACATTATCCTCAAGTGGTAGAGACATGAGTGTCTCAAAATTAAGTGGGCGCAAGCAGGTAAGTAAAGTGGTAAAGTCTTACCATTAGATCATTTAAGGACCAAGC
2401
2521 TTTATCAAAGCCCACTTCTTATTTGATGTTCTGAATGTTATCGCGCGGGCTATGTTGATTATGATCTAGTACCCCTGGTAACACCGAGGAAGAAAGGTACGAAACGCAAGATTGGC
2521
2641 TATTCTATTGTAGAAAGTTGGTGCTTTGATTTGGTTGGTGCCAGATTAACCAAGGTTTCGTGCAAGATTAATTAATTAATTTTTCGTTTCGTTACGTTTGAACAAATGAGC
2641
2761 CAACAAACAACTTTCGTAACCTGTTTTCTACTACGTTTCTTGTTCATGCTCGGTTATACGATTGGTTAAGTGTACAACAATATATAGCCCTCAAGTATTTCGAAGTTTTTAATATGT
2761
2881 AATAATAGATTTCAACTTCCACCTACTTTTATTCGTGATTACTGAGCCATTGTAATAAGTCCCAACGCTCAATGATTCCGTTGCTGCTTCTATCATGCTCTACTGGATTAAAAAT
2881
3001 CGTAAATAAACTACTTTAATAGCCATATATCCTGTGAACTTTTTTTCATATACATCCAAGGTTCTTTGAAATATGCAGAAATGTACGTGATAGTTAAGCTTTTAGGCATCGAACATTTC
3001
3121 CGGACAATGACCCACATCCAGATGGGCACTCATCAACATCTCCACCATATCCT

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FIG. 2 Nucleotide and predicted amino-acid sequence of *SAC6*. Underlined nucleotides are splicing consensus sequences in the intron²³.

METHODS. To sequence *SAC6*, it was first necessary to isolate a complete, wild-type copy of the gene; previously, complete mutant and incomplete wild-type copies had been obtained^{1,3}. To this end, plasmid pRB1276 (ref. 1), containing an incomplete copy of wild-type *SAC6* in Ylp5, was integrated into the *SAC6* locus of DBY2057 (*MATa ura3-52 SAC6⁺*). Genomic DNA was isolated, cut with *AatII*, and circularized by ligation. Amp^r transformants were selected in *Escherichia coli*, and plasmid DNA containing the whole of *SAC6* and most of Ylp5 was isolated. The *SacI*-*NcoI* fragment, containing the complete *SAC6* gene³, was subcloned into pUC118 and pUC119 (ref. 24). Two sets of overlapping unidirectional deletions were generated as described previously²⁵ except that exonuclease VII was used instead of S1

nuclease. Overlapping sequence of both strands was obtained between nucleotides 325 and 2,987 (Fig. 2). The remaining sequence was obtained on one strand only. Preliminary analysis of the *SAC6* sequence indicated there was an intron near the 5' end of the gene. To locate the splice junctions precisely, poly(A)⁺ RNA (kindly provided by S. Mayer) from strain D273-10B (ref. 26) was used to generate cDNA as described²⁷. Primers designed to flank the putative intron were used to amplify this region of the cDNA by the polymerase chain reaction (PCR). The primer at the 5' end corresponded to nucleotides 694 to 710 of the sequence shown, and the primer at the 3' end corresponded to nucleotides 1,005 to 1,038. The PCR product was then cloned into plasmid pRS416 (ref. 28) and sequenced, confirming the existence of the intron, and the indicated splice junctions.

at low temperature and cortical actin acts to strengthen actively growing regions of the cell surface that otherwise might be weakened during cell growth, as has been suggested for tip extension in pollen tubes²¹. Consistent with this possibility, we have noted in processing these cells for immunofluorescence microscopy that they appear more fragile than wild type, even

when grown at 23 °C.

Our evidence is most consistent with the view that the fimbrin homologue Sac6p is an actin-filament bundling protein that acts in yeast to 'fine-tune' the structure of the actin cytoskeleton, making it possible for the actin cytoskeleton to function optimally even when the cell is stressed. □

a

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Chicken fimbriin      MENNVTTISREELEELREAFNKKIDIDNSGYVSDYELQDLFKEASLPPLGYKVREIEKIFAVTDSNKGKINFEFVSLIQ
Yeast Sac6p          MNIVKLQRKFPILTQEDLFSTIEKFRAIDDDKGWVEKQQALEAVSKDGA--TYDEARETLKHVGVDASGRVLEDDYVGLVAKLRESKTGAAPQTTFN---V
Human L-plastin      MARGSVSDEEMMELREAFKVDTDGNGYISFNEIENDLFKAACLPPLGYRVREIYENLMATGDLQDGRISFDEFIKIFH

f ELKSKDVSKSYRKSINKKLGITAGTSSISTEGTQHSYSEEEKYAFVNWINKALQDDPCKHILPMNPDSASLFKSLADGILLCKMNFSPQDPTIDERAIN---KKKLTPTTISENLN
s .....
p GLKSTDVAKTRKAIKKKGGICATGGTSSQSSVGTQHSYSEEEKYAFVNWINKALENDPDCRHVPMNPNTNDLFNAVGDGIVLCKMINLSVDPDTIDERTIN---KKKLTPTTISENLN

f LALNSASAIGCTVNVNIGSQDLOEGKPHIVLGLIWOIKVGLFADIELSRNEALIALNEGEELDQIMKLSPEELLRWVNYHLANAGW-QKISNFSQDIRDSRAYYHLNQIAPKGGDDFD
s .....
p LALNSASAIGCHVNVNIGARDLKEGKPYVLGLIWOIKVGLFADIELSRNEALIALREGESLEDLMKLSPEELLRWVNYHLNAG-CNKIGNFSTDIDKSKAYYHLEQVAPKGDDEG

f EIHVEIDFSGFNDKNDLRAECMLQQADKLGCRQFVTPADVAGNPKNLAFVANLNTYPAHKK-PDNSSYDLTLLEGESN-EERTFRNMNSLGVSPYVNHLYSDLSDALIIFQLYE-
s ---ALCSKAPLQTTDLMERAEQVLQNAEKLDCKRYLTPSSLVAGNPKNLAFVAHLNTHPGLEPIQEEKPEIEEFDAEGEREARVFTLWLSLDVDPVVISLFDLKDGLILLQAYEK
p VPAAVIDMSGIREKDDIQRAECMLQQAERLGCRCQFVTATDVVRGNPKNLAFIANLFWRYPAHKK-PENQDIDWGALEGE-TREERTFRNMNSLGVNPRVNHLYSDLSDALVIFQLYEK

f MTRVPVDWTHVNRKPYPLLG-GNMKKIENCNYAVELGKTKAKFSLVGIAGHDLNEGNTLTTLALIWOLMRRYTLNVLSDL-GEGEKVNDIEIIKWVNYQLANANKKTSITSFKDKSISTS
s .....
p IK-VPVDWNRVNRKPPYKLG-GNMKKIENCNYAVELGKNQAKFSLVGIGGODLNEGNTLTTLALIWOLMRRYTLNILEEI-GGGQKVNDIIVNVWNETLREAEKSSISSFKDKPKISTS

f LPVLDLIDAIAPKAVRQEMVKREDLSYQDKLNNAKYAISSVARKIGARIYALPDDLVEVPMKMMVTVFACLMGRGLNKKI
s .....
p LPVLDLIDAIQPGSINYDLTKTENLNDDEKLNNAKYAISSMARKIGARVYALPEDLVEVPMKMMVTVFACLMGKGMKR

```

b

Sac6p	231	VNVHSEDIIEGREHILGLIWOIIRH	257
Fimbrin	210	VVNIGSQDLQEGKPHIVLGLIWOIIRH	236
L-plastin	208	VVNIGAEELKEGKPYIVLGLIWOIIRH	234
β -spectrin	126	LENIGSHDIVDGNASLNLGLIWTIIR	152
ABP-120	89	LVGIGAEIDIVSOLKILGLIWTIIR	115
Dystrophin	91	LVNIGSTDIVDGNHKLTLGLIWNIIIR	117
α -actinin	107	LVSIGAEIIVDGNVMTLGMIIWTIIR	133
ABP-280	121	LVSIDSKAIVDGNKILGLIWTIIR	147
Sac6p	493	LVGIEGSDIVDGNKLLTLGLIWOIMRR	519
Fimbrin	477	LVGIAGHDLNEGNTLTTLALIWOLMRR	503
L-plastin	475	LVGIGGODLNEGNTLTTLALIWOLMRR	501

FIG. 3 Sac6p homologies. *a*, Alignment of chicken fimbriin⁴, yeast Sac6p and human L-plastin^{6,7} sequences. Double dots, identities; single dots, similarities according to the following groupings: D, E, S, T; I, L, V, M; A, G, F, W, Y; N, Q; H, R, K; C; P. Underlined amino acids are the two putative actin-binding domains shown in *b*. *b*, Putative 27 amino-acid actin-binding domains of indicated actin-crosslinking proteins. Dark shade, identities; light shade, similarities (groupings as in *a*). Dark shading for groups of four or more identities; light shading for residues similar to the group of identical residues. Numbers indicate residue positions. References to amino-acid sequences: chicken fimbriin, ref. 4; human L-plastin, refs 6, 7; *Drosophila* β -spectrin, ref. 9; *Dictyostelium* ABP120, ref. 8; human dystrophin, ref. 11; human α -actinin, ref. 12; human ABP280, ref. 10. *c*, Potential EF hands of Sac6p and chicken fimbriin⁴ compared with EF hands (helix-loop-helix I, in each case) of bovine brain calmodulin and chicken skeletal troponin C (see

C	Helix	Loop	Helix	
		X Y Z-Y-X -Z		
15	QEDLFSTIEKFRAI	DLDDKGWVE	KQQALEAVSKD	Sac6p
50	DATYDEARETLKHV	GVDASGRVE	LDDYVGLVAKL	Sac6p
10	REELEELREAFNKI	DIDNSGYVS	DYELQDLFKEA	Fimbrin
50	YKVRREIEKIFAVT	DSNKGDKIN	FEFVSLIQEL	Fimbrin
6	EEQIAEFKEAFSLF	DKDGDGTIT	TKELGTVMRSL	CaM
16	EEMIAEFKAAFDME	DADGGGDIS	TKELGTVMRML	TnC

ref. 13). Following the convention for calcium-binding sites, the X, Y, Z, -X, -Y and -Z positions correspond to residues that coordinate with calcium in calmodulin and troponin C (ref. 13). Numbers indicate residue positions.

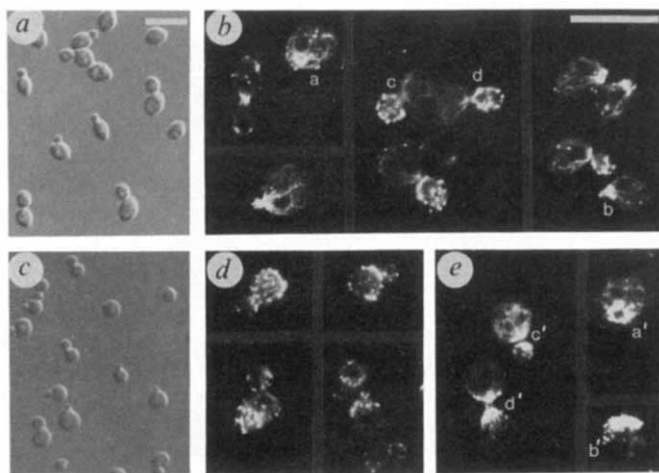


FIG. 4 Phenotypes of strains lacking Sac6p. Differential interference contrast (DIC) and fluorescence micrographs of wild-type strain AAY1043 (a, b) and *sac6*-null mutant strain AAY1042 (c–e) cells grown at 23 °C. For fluorescence microscopy, cells were stained with anti-actin antibodies (b, d, e). Scale bar, 10 μ m.

METHODS. Generation of the *sac6* null mutation. A *LEU2*-containing fragment was placed between *SAC6*-flanking regions obtained by PCR amplification of nucleotides 143–710 and 2,759–3,120 (fig. 2) and inserted into pUC218 (Genentech). The *LEU2* gene bordered by the *SAC6*-flanking sequences was cut out of the plasmid and used to transform diploid yeast strain TPS507 (*Mata/MAT α ade2/+ his3/his3 leu2/leu2 lys2/lys2 ura3/ura3*). *Leu*⁺ transformants were selected, and the replacement of one of the copies of *SAC6* by *LEU2* in one isolate (AAY1069) was confirmed by Southern blotting of genomic DNA. Further confirmation was obtained by tetrad analysis. Thus, 14/14 tetrads derived from AAY1069 showed 2:2 cosegregation of temperature sensitivity and the *LEU2* marker. Southern-blot analysis of genomic DNA from all four segregants from each of three tetrads showed the expected pattern of restriction fragments: the *Leu*[–] Ts[–] segregants had the wild-type pattern, and the *Leu*⁺ Ts[–] segregants the replacement pattern (not shown). Further, immunoblot analysis of each of the 12 segregants showed that Sac6p was present in each of the six Ts⁺ *Leu*[–] segregants, but absent from the six Ts[–] *Leu*⁺ segregants (not shown). *SAC6*⁺ segregants derived from AAY1069 were crossed to each other to generate strain AAY1043 (*MAT α /MAT α SAC6⁺/SAC6⁺ lys2/lys2 ade2/+ leu2/leu2 ura3/ura3 his3/his3*). *sac6::LEU2* segregants derived from AAY1069 were crossed to AAY220 (*MAT α leu2*) or AAY221 (*MAT α leu2*), and segregants from these crosses were mated in various combinations to generate strains AAY1040 (*(MAT α /MAT α ura3/ura3 his3/+ leu2/leu2 sac6::LEU2/sac6::LEU2 ade2/+)*), AAY1041 (*(MAT α /MAT α leu2/leu2 sac6::LEU2/sac6::LEU2 lys2/+ ura3/+)*), and AAY1042 (*(MAT α /MAT α sac6::LEU2/sac6::LEU2 lys2/+ ade2/ade2 leu2/leu2 ura3/+)*). These four strains were used in the phenotypic analyses described in the text. Immunofluorescence microscopy was done as described previously²⁹, using affinity-purified polyclonal anti-yeast actin antibodies². Fluorescence micrographs were obtained using hypersensitized Kodak Technical Pan 2415 film (Lumicon; see ref. 29).

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A 'molten-globule' membrane-insertion intermediate of the pore-forming domain of colicin A

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THE 'molten' globular conformation of a protein is compact with a native secondary structure but a poorly defined tertiary structure^{1,2}. Molten globular states are intermediates in protein folding and unfolding^{3–5} and they may be involved in the translocation or insertion of proteins into membranes⁶. Here we investigate the membrane insertion of the pore-forming domain of colicin A, a bacteriocin that depolarizes the cytoplasmic membrane of sensitive cells^{7–9}. We find that this pore-forming domain, the insertion of which depends on pH (refs 10, 11), undergoes a native to molten globule transition at acidic pH. The variation of the kinetic constant of membrane insertion of the protein into negatively charged lipid vesicles as a function of the interfacial pH correlates with the appearance of the acidic molten globular state, indicating that this state could be an intermediate formed during the insertion of colicin A into membranes.

Models based on the X-ray structure of the colicin A pore-forming domain have been proposed for the first step of its electrostatic membrane insertion mechanism and for the resulting structure of the inserted monomer in the absence of membrane potential^{12,13}. These are largely supported by experiment^{13–17}, but the intervening stage is not well understood. By analogy with enzyme reactions, the kinetics of membrane insertion of colicin A into lipid vesicles should strictly depend on the proportion and the energy state of this insertion intermediate.

We have previously shown that the three tryptophan residues of the pore-forming fragment become accessible to the lipid phase on membrane insertion¹¹. Addition of brominated dioleoylphosphatidylglycerol (Br-DOPG) liposomes to the protein in solution results in an exponential decrease of the tryptophan fluorescence due to quenching by the bromine in the middle of the phospholipid fatty acyl chain. We have determined the kinetic constant of insertion of the pore-forming domain into vesicles by recording the quenching of the intrinsic fluorescence by Br-DOPG as a function of time (Fig. 1). Transformation of the initial fluorescence decay according to the equation given in Fig. 1 legend, as shown in the inset of Fig. 1, gives a straight line, so it is reasonable to assume first-order kinetics, at least for the initial period of the insertion. The slope of the straight

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line, k , is thus the apparent rate constant for the insertion of the pore-forming domain into Br-DOPG vesicles.

Low pH dramatically increases the affinity of the pore-forming domain of colicin A and its fragment for lipid monolayers or liposomes^{10,18}. Figure 2 shows that k for both colicin A and its thermolytic fragment is also strongly pH-dependent. From polarization anisotropy data at pH values above 5.5 the rate of insertion is very low and binding is poor (results not shown). Below pH 5.5, k starts to increase as the pH is lowered and levels off below pH 4.2. Similar results were obtained with full-length colicin A and its C-terminal domain. The apparent pK governing the kinetics of insertion is around pH 4.8 in both cases (slightly lower than the value in lipid monolayers¹⁰), confirming that colicin A interacts with lipids mainly through its pore-forming domain.

If the changes in colicin A affinity for lipid vesicles below pH 5 are due to a pH-dependent conformational change of the protein, then the change should be revealed by fluorescence and circular dichroism (CD) spectroscopy, which we use here to analyse secondary structure and the environment of aromatic residues. CD measurements on colicin A pore-forming fragment showed that the ellipticity at 222 nm, characteristic of the α -helical conformation, decreases by only 15% between pH 7.0 and pH 2.0 (Fig. 3a), indicating that a native-like secondary

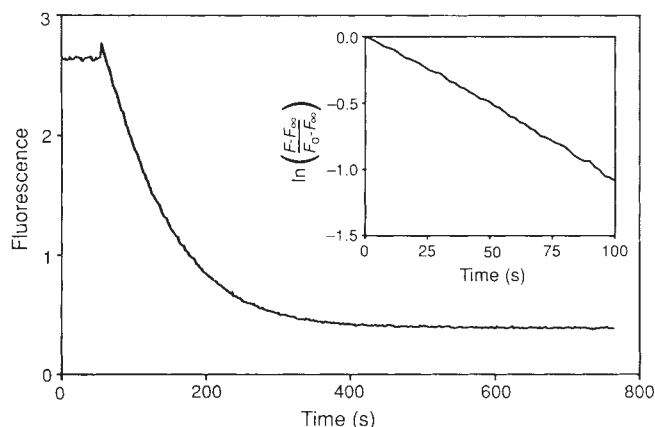


FIG. 1 Kinetics of membrane insertion of the colicin A thermolytic fragment measured by quenching of intrinsic protein fluorescence by brominated dioleoylphosphatidylglycerol (Br-DOPG) vesicles. Fluorescence is given in arbitrary units. The results can be interpreted in terms of a one-step association/dissociation process³⁰, whereby the change in the intrinsic fluorescence of the colicin A thermolytic fragment after addition of brominated liposomes is related to the free colicin concentration, $[C_f]$, by $[C_f] = [C_{tot}]/((F_t - F_\infty)/(F_0 - F_\infty))$ where $[C_{tot}]$, F_t , F_0 and F_∞ are the total colicin concentration where $[C_{tot}]$, F_t , F_0 and F_∞ are the total colicin concentration and the fluorescence intensity at times t , 0 and 'infinity' respectively. The linearity of the inset plot shows that the reaction is pseudo first order in free colicin concentration under these conditions. At this lipid-to-protein molar ratio, all colicin molecules are bound to the vesicles at $t = \infty$ ($R_f = 500$), as shown by ultracentrifugation and fluorescence anisotropy (data not shown). This indicates that the rate of dissociation is negligible and the observed rate is solely that of association. Hence a plot of $\ln((F_t - F_\infty)/(F_0 - F_\infty))$ versus time gives a straight line, whose slope gives the apparent rate constant for the insertion of the protein into the liposome (lipids concentration is a constant at time $t \rightarrow 0$). Note that k is a phenomenological constant which can be expressed as a function of the true kinetic constants according to ref. 30 or other kinetic models.

METHODS. The brominated phospholipid was synthesized as described³¹. Br-DOPG from stock solution was dried in a rotary evaporator and lipid film left overnight under vacuum before suspending in 50 mM Tris-acetate, pH 5.0. Liposomes were formed by bath sonication at room temperature. To 400 μ l of a solution of colicin A thermolytic fragment ($A_{280} = 0.018$) were added 15 μ l Br-DOPG vesicles (10 mg lipid per ml) and the change in the intrinsic fluorescence emission was measured in real time. Fluorescence spectra were recorded at 30 °C in an SLM 8000 spectrofluorometer in ratio mode with bandwidths of 4 nm for excitation and emission, and using the same precautions as before^{11,15}.

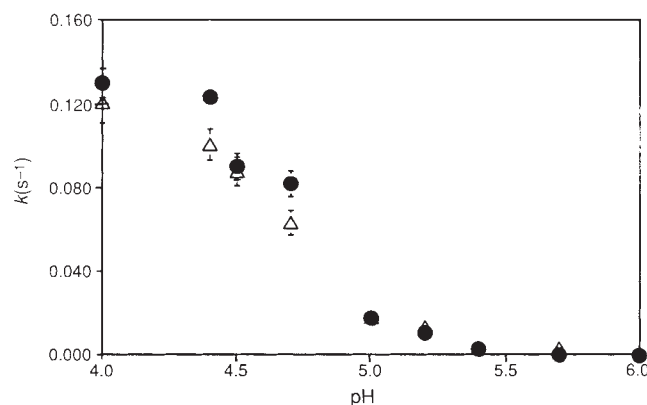


FIG. 2 Effect of pH on the rate of insertion of colicin A (Δ) and its thermolytic fragment ($\bullet$) into Br-DOPG liposomes. Colicin A, its thermolytic fragment, and Br-DOPG vesicle solutions were prepared in 50 mM Tris-acetate buffer at different pHs. The rate of insertion at each pH was calculated as described in the legend to Fig. 1 (also see text).

structure persists even at low pH. Moreover, the intrinsic tryptophan fluorescence spectrum of the thermolytic fragment in solution had a constant maximum emission wavelength between pH 2 and pH 7 (λ_{max} , 332 nm), showing that the accessibility to water of the buried tryptophans does not increase at acidic pH. But the CD spectrum in the near ultraviolet (determined by the conformation of aromatic residues) showed a marked difference: at pH 7.0, there is a strong ellipticity, with three major peaks (Fig. 3b) which are indicative of fixed orientations of aromatic residues in the protein in solution. The recently refined structure of the fragment has revealed that these aromatic residues form a well-defined cluster made up of Trp 86 stacked against Tyr 161 and Trp 130, which packs perpendicular to Tyr 125 (ref. 13). At pH 2.0 the CD spectrum in the near ultraviolet has almost completely collapsed (Fig. 3b).

These results indicate that below pH 4, the thermolytic fragment adopts, as do many other proteins, the features of the molten globule state^{1,2,19,20}: a substantial secondary structure, no native tertiary structure and a compact water-excluding core (deduced from the maximum emission wavelength of tryptophan). Figure 3c shows the dependence of the molten globular state on pH, as evidenced by the variation in ellipticity at 294 and 222 nm.

The midpoint of the native to molten globule transition is much lower (pH 2.7) than the pK that governs the kinetics of insertion of the colicin into membrane vesicles (Fig. 2). But it should be pointed out that on negatively charged DOPG vesicles the surface pH differs from that of the bulk^{21,22}, and so conditions are not quite the same as those in solution. The negatively charged polar heads give rise to an electrical surface potential (Ψ_0) which in turn decreases the surface pH; Ψ_0 may also influence the local anion concentration which itself affects the acidic molten globule transition⁴, but this effect is likely to be marginal at the low ion concentrations used here. Although Ψ_0 can be calculated from the surface density of the lipids using the Gouy-Chapman theory²¹, the calculation may not hold for vesicles made up of only negatively charged phospholipids. We therefore determined Ψ_0 values at different pHs (Fig. 4 legend). At the low ionic strength used here, the Δ pH between bulk and the vesicle surface can be as large as 1.62 pH units (bulk is pH 5.0), so this effect must be taken into account. Calculation of the corresponding Ψ_0 using the Gouy-Chapman theory gives a Δ pH of 2.7: this overestimate may be a result of the assumption that a planar bilayer has a continuous rather than discrete surface charge^{21,23}. The kinetic constant of colicin binding versus the measured interfacial pH (Fig. 4) can now be plotted, together with the concentration of the molten globular state (calculated from the change of ellipticity at 294 nm) versus bulk pH (see

Fig. 4 legend). This plot shows a clear correlation between the increase of k and the appearance of the molten globular state.

Our results support the following scheme for the pH-dependent mechanism of insertion of colicin A into membranes. The structure of the C-terminal fragment of colicin A is a three-layered structure composed of 10 α -helices, two of them forming a hydrophobic helical hairpin buried in the soluble protein¹². An electrostatic interaction between the ring of eight positively charged residues on one face of the protein and the negatively charged lipid interface orients the molecule with its hydrophobic hairpin perpendicular to the membrane plane^{12,13}. The local pH at the interface induces the molten globular state transition. This transition to a flexible conformation lowers the energy required to unmask the hydrophobic hairpin and allows the structure to open like an umbrella during membrane insertion. The helices present in the soluble structure are preserved in the membrane-bound form^{10,11} and so the molten globular state, by preserving secondary structure (in contrast to complete unfolding), provides the best pathway for minimizing the energetic cost of making a compact protein structure competent for membrane insertion. Such a mechanism seems to be common to all pore-forming colicins. The pore-forming domain of colicin E1 undergoes a conformational change below pH 4.0 into a translocation-competent state which, because its sensitivity to proteases and fluorescence-quenching agents is increased while its radius of gyration²⁴ and α -helical content are unaltered¹⁴, is most probably a molten globule state.

We have demonstrated a direct correlation between the kinetics of membrane insertion and the assumption of a molten globular conformation. This result is relevant to the translocation of toxins through the membrane of acidic organelles that is triggered at low pH (refs 25, 26). In the case of diphtheria toxin^{27,28} the difference in stability of the A and B subunits could mean that only subunit A, the translocated unit, can

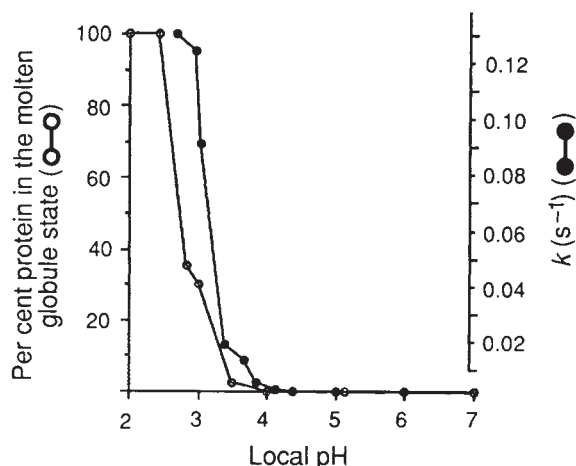


FIG. 4 Variation of k , the kinetic constant of membrane insertion, and the percentage of protein in the molten globular state as function of local pH at the negatively charged lipid/water interface. Surface pH was calculated from the measured surface potential (Ψ_0) of the vesicles at different pHs according to $pH_{\text{surface}} = pH_{\text{bulk}} + F\Psi_0/2.303RT$.

METHODS. Ψ_0 at different pHs was evaluated fluorometrically by measuring the surface-potential-dependent binding of 2-(*p*-toluidinyl)naphthalene-6-sulphonate (TNS) to DOPC (dioleoylphosphatidylcholine) and DOPG vesicles as described²³. The net fluorescence is proportional to the number of TNS molecules adsorbed to the membrane. The ratio of the probe molecules adsorbed to a charged surface (DOPG) to those adsorbed to a neutral surface (DOPC) are related to Ψ_0 by $\Psi_0 = (RT/F) \ln(f/f_0)$, where f and f_0 are the net fluorescent intensities of TNS adsorbed to DOPG and DOPC vesicles respectively. Liposome preparation and fluorescent measurements were as described for Fig. 1; excitation and emission wavelengths were 321 and 446 nm respectively.

undergo the molten state transition at the pH of acidic organelles. Also, the aggregation and pH-independent insertion into the membrane of human complement C9 proceeds through an intermediate analogous to one of the thermally unfolded stages observed by differential scanning calorimetry²⁹. □

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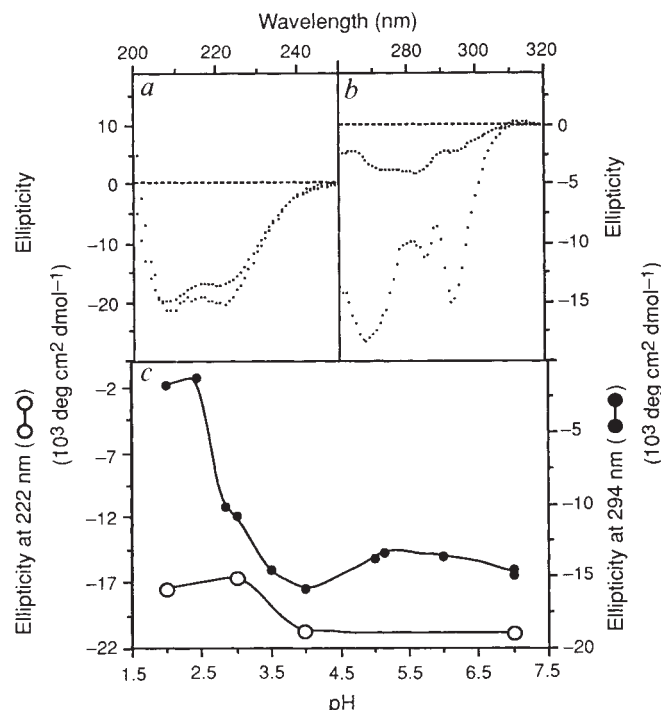


FIG. 3 Acid-denaturation curves of colicin A thermolytic fragment. The mean residue ellipticity in the far ultraviolet (a) and the molar ellipticity in the near ultraviolet (b) of the colicin A thermolytic peptide at pH 7 (lower spectra) and pH 2 (upper spectra). c, Influence of pH on the ellipticity at 222 nm and 294 nm. The thermolytic fragment was dissolved in 30 mM Na_2HPO_4 and the pH adjusted with citric acid. Spectra were acquired on a Jobin Yvon CD6 instrument at 25 °C using protein concentrations of 0.1–0.2 mg ml⁻¹ (a) and 0.5–3 mg ml⁻¹ (b) and cell pathlengths of 1 mm and 2 mm respectively.

New protein fold revealed by a 2.3-Å resolution crystal structure of nerve growth factor

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NERVE growth factor (NGF)¹ is a member of an expanding family of neurotrophic factors (including brain-derived neurotrophic factor² and the neurotrophins^{3,4}) that control the development and survival of certain neuronal populations both in the peripheral and in the central nervous systems⁵. Its biological effects are mediated by a high-affinity ligand–receptor interaction and a tyrosine kinase signalling pathway^{6,7}. A potential use for NGF and its relatives in the treatment of neurological disorders such as Alzheimer's disease⁸ and Parkinson's disease⁹ requires an understanding of the structure–function relationships of NGF. NGF is a dimeric molecule, with 118 amino acids per protomer. We report the crystal structure of the murine NGF dimer at 2.3-Å resolution, which reveals a novel protomer structure consisting of

three antiparallel pairs of β strands, together forming a flat surface. Two subunits associate through this surface, thus burying a total of 2,332 Å². Four loop regions, which contain many of the variable residues observed between different NGF-related molecules, may determine the different receptor specificities. A clustering of positively charged side chains may provide a complementary interaction with the acidic low-affinity NGF receptor. The structure provides a model for rational design of analogues of NGF and its relatives and for testing the NGF-receptor recognition determinants critical for signal transduction.

Murine NGF (β -NGF) was purified from the submandibular glands of adult male mice according to Darling and Shooter¹⁰. Crystals of β -NGF were obtained from 10 mM phosphate buffer, pH 6.8 with 2-methylpentane 2,4-diol as precipitant. The space group is P6₃22 with unit cell parameters $a = b = 56.48$ Å and $c = 182.39$ Å, containing a single β -NGF subunit per asymmetric unit¹¹. Table 1 summarizes the crystallographic data. The structure was solved by the multiple isomorphous replacement with anomalous scattering (MIRAS) method using four heavy-atom derivatives. The initial map at 3.3 Å resolution was improved by applying a solvent-flattening procedure¹² allowing the location and fitting of 80% of the side-chain densities from the known amino-acid sequence¹³ (Fig. 1a). There were breaks in the main-chain density in the regions 43–48 and 76–79. The heavy-atom sites gave useful support for the interpretation of the solvent-flattened map (Table 1). Electron density maps calculated with coefficients $2F_o - F_c$ and $F_o - F_c$ either after simulated annealing with X-PLOR¹⁴ of a model in which all side chains were truncated to alanine (glycines remaining as glycine) or after omission of 8–10-residue stretches confirmed the interpretation.

Several regions of the structure were poorly determined both in the MIRAS and the solvent-flattened maps. No density was observed for residues 1–10 (the amino terminus) and residues 112–118 (the carboxy terminus). Both of these regions are susceptible to proteolytic modification¹⁵ and are likely to be both

TABLE 1 Statistics for crystallographic analysis

Dataset	Resolution (Å)	Images/filmpacks (source)	Unique reflections (% data)	R_{sym}^*	Average isomorphous difference
Native	2.3	60 (DESY)	7,522 (90.3%)	6.6%	—
K ₂ Pt(NO ₂) ₄	3.1	18 (SRS)	3,213 (90.2%)	6.6%	17.0%
NaAuCl ₄	3.1	12 (SRS)	2,922 (82.9%)	6.5%	19.2%
EtHgPO ₄ ²⁻	3.1	18 (SRS)	3,270 (91.8%)	9.8%	14.8%
UO ₂ SO ₄	3.5	12 (SRS)	2,063 (86.4%)	9.1%	17.4%

Dataset	Number of sites	$R_{\text{centric}}^{\dagger}$	Heavy atom parameters (occ, x, y, z)	r.m.s. $f_h/E^{\ddagger}$	Binding site on NGF subunit
K ₂ Pt(NO ₂) ₄	1	63.8%	0.161 0.014 0.748 0.074	1.06	His 75
NaAuCl ₄	1	65.8%	0.193 0.684 0.714 0.075	1.39	His 84
EtHgPO ₄ ²⁻	1	68.0%	0.143 0.586 0.682 0.038	1.04	His 84
UO ₂ SO ₄	2	64.7%	0.107 0.550 0.182 0.015 0.085 0.573 0.584 0.028	1.02	Glu 35 Asp 105

Diffraction data were collected using synchrotron radiation and photographic film at the SERC Daresbury Laboratory (SRS) and the image plate data-collection system EMBL outstation of DESY, Hamburg (DESY). These data were processed using the program MOSFLM³⁴ as modified by H. Terry for the image plate system. All other crystallographic calculations were done using the CCP4 program suite (SERC collaborative computing project number 4). The space group and absolute configuration were determined by a series of cross-phased difference Fourier syntheses using the anomalous contribution of the heavy-atom derivatives, which indicated P6₃22 to be the correct space group. Heavy-atom parameters refined were x, y, z and occupancy; temperature factors were fixed at $B = 20$ Å². A 3.3-Å map was calculated using the isomorphous and anomalous contributions to give a mean figure of merit of 0.64 for 2,750 reflections between 18.0 and 3.3 Å. Solvent flattening was done according to Leslie¹² with an estimated solvent content of 55% and iterated for five cycles. The polypeptide chain was fitted using the program FRODO³⁵ and by referring to 'mini-maps' of both the MIRAS and solvent-flattened maps plotted on polyester sheets. The molecular model was refined by a combination of simulated annealing using the program X-PLOR¹⁴ and restrained least-squares refinement as implemented by the program RESTRAIN³⁶. The current R -factor is 23.6% for all reflections between 8.0 and 2.3 Å. The root-mean square deviation from ideal bond lengths is 0.04 Å. All but one non-glycine residues lie in the 'allowed' regions of the Ramachandran plot, the exception being residue Asn 45. The heavy-atom derivatives for NGF are called NaAuCl₄, sodium gold (III) tetra-chloride; EtHgPO₄²⁻, ethyl mercury phosphate; Pt(NO₂)₄, platinum (II) tetra-nitrite; UO₂SO₄, uranyl sulphate.

^{*} $R_{\text{sym}} = \sum |I_{\text{obs}} - \langle I \rangle| / \sum \langle I \rangle$.

[†] $R_{\text{centric}} = R$ -factor for centric reflections.

[‡] f_h , Calculated heavy-atom structure factor. E, Residual lack of closure errors. The average isomorphous difference was calculated between the derivative and native structure factor amplitudes.

flexible and solvent accessible. The preparation of β -NGF contained a proportion of β -NGF with residues 1–8 deleted (estimated at 25%) and β -NGF with residue 118 deleted (estimated at 10%). In addition several loop regions had poor definition in the electron density (residues 43–48 and 76–79). This and an average temperature factor of $34 \text{ \AA}^2$ suggest a highly flexible structure similar to other growth factors.¹⁶

In agreement with predictions based on Raman spectroscopy¹⁷ the β -NGF subunit consists of a predominantly β -structure domain. There are seven β strands, which contribute to three antiparallel pairs of β strands (Fig. 2a). The large twist in each pair of β strands and the interface between the A'-A'' and B-C double-stranded sheets allow the formation of a β -NGF subunit hydrophobic core, which includes residues Val 36, Val 38, Phe 53, Ile 71, Ala 89, Ile 102 and Ile 104 (Fig. 2b). Additional subunit core is contributed by the three disulphide bonds, which are closely packed and show some similarity to those of the neurotoxin family^{18–20}; however, no good superposition was possible with any of the available neurotoxin structures.

A high degree of sequence conservation of the structurally important residues in β -NGF between members of the NGF family (Fig. 1c) suggests that the same overall three-dimensional structure is adopted. In addition to the disulphides, several side-chain to side-chain hydrogen bonds are likely to be absolutely conserved in all the sequences. These include Asp 16 to Arg 69, Ser 19 to Thr 56, Lys 25 to Glu 55, Asp 72 to His

75, and Thr 91 to Arg 100. Several important hydrogen-bonding sidechains are buried in the β -NGF subunit, including Asp 30 which has a role in the β -hairpin turn at residues 30–33, Ser 78 and Thr 91 which hydrogen bonds to Arg 100. Gln 51 is likely to be a critical residue for the stability of the NGF protomer as it is inaccessible to solvent and hydrogen bonds to the main chain amide function of residue 90 and the carbonyl oxygen of residue 39.

The β -NGF subunit (roughly $60 \times 25 \times 15 \text{ \AA}$) has a surface area of $7,229 \text{ \AA}^2$. In the dimer, two protomers assemble about a 2-fold axis ($x, \bar{x}, z = 1/12$) with their long axes roughly parallel and their flat faces in contact at the dimer interface. The surface area of the dimer is $12,126 \text{ \AA}^2$ indicating that about $2,332 \text{ \AA}^2$ are buried in the dimer. The (principally hydrophobic) contacts are localized in three regions. At the top of the view in Fig. 2b, Phe 49 of one subunit and Trp 99 of the 2-fold related subunit are packed together; in the middle Trp 21, Tyr 52 and Phe 54 of one 'edge' of the subunit pack against Phe 86 and Phe 101 of the other subunit in the dimer; towards the base of the subunit, residues Phe 12 and Trp 76 of the other subunit interact, in addition to contributions from residues Val 14, Ile 71, Ala 107, Val 109 and Val 111. Two polar residues Thr 85 and Thr 106 are also buried in the dimer interface. The hydrophobic nature of the dimer interface of β -NGF would explain the tight association constant (K_a) of 10^{13} M^{-1} for the two subunits. The hydrophobic free energy contributed by this interface is

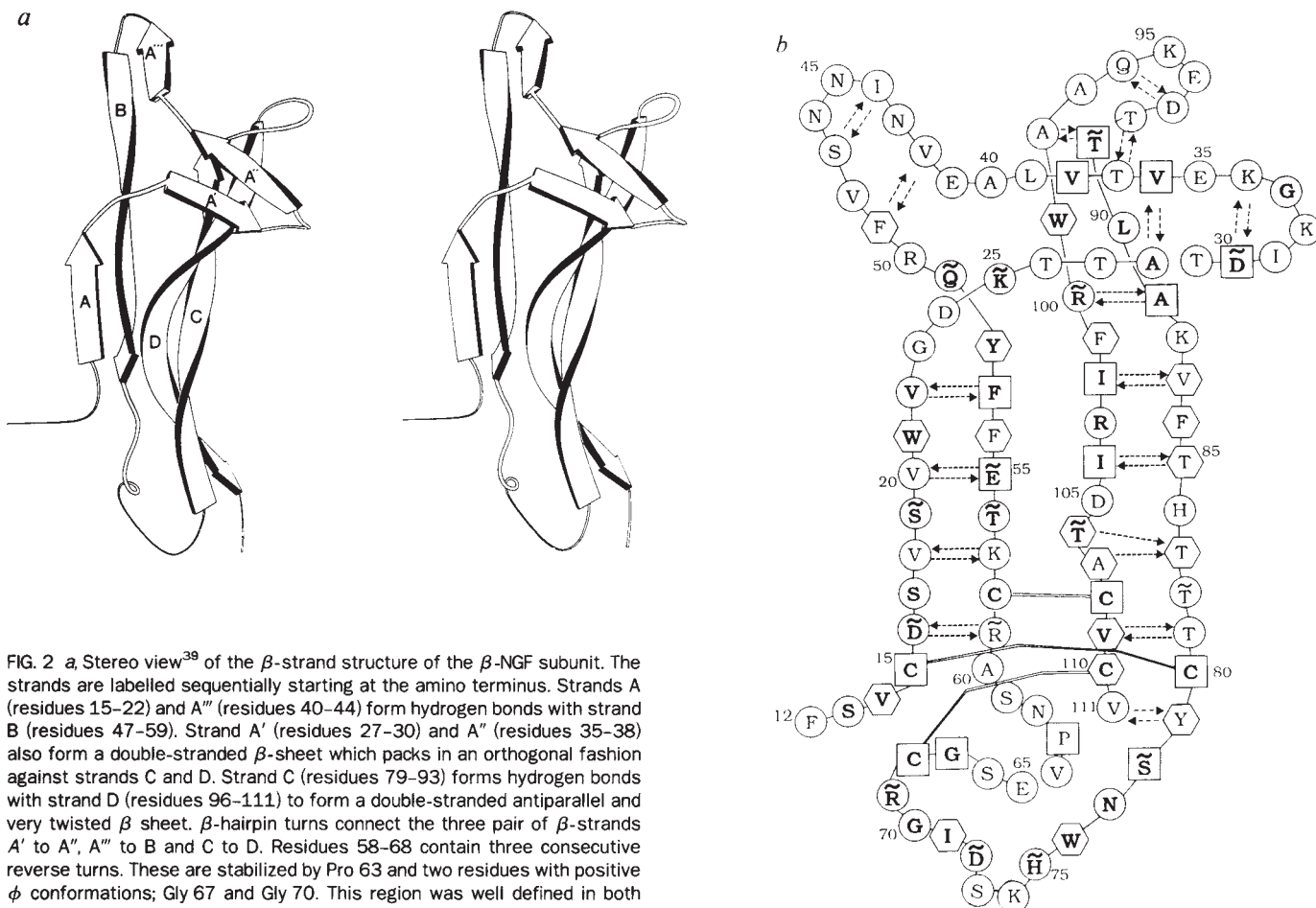
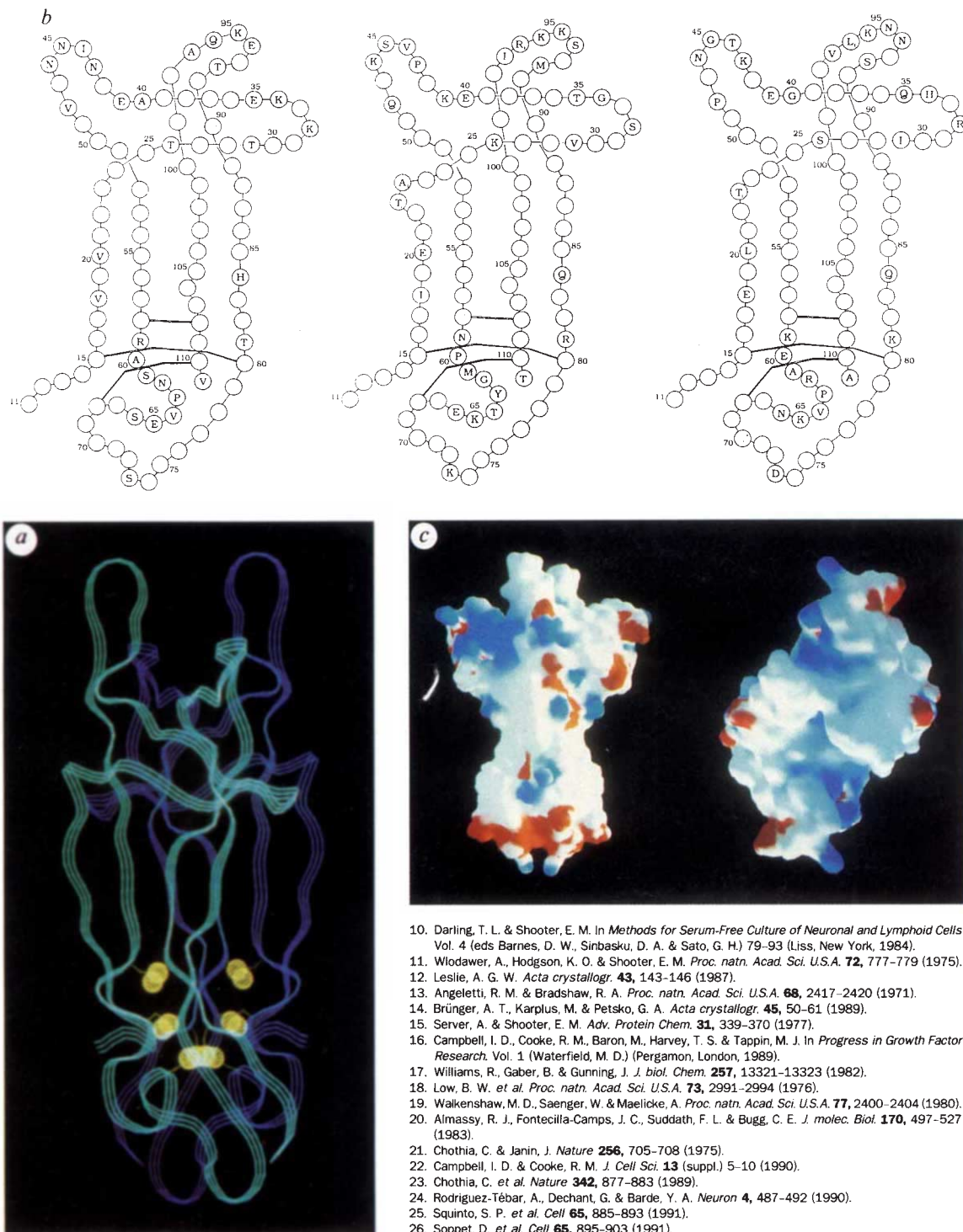


FIG. 2 a Stereo view³⁹ of the β -strand structure of the β -NGF subunit. The strands are labelled sequentially starting at the amino terminus. Strands A (residues 15–22) and A'' (residues 40–44) form hydrogen bonds with strand B (residues 47–59). Strand A' (residues 27–30) and A''' (residues 35–38) also form a double-stranded β -sheet which packs in an orthogonal fashion against strands C and D. Strand C (residues 79–93) forms hydrogen bonds with strand D (residues 96–111) to form a double-stranded antiparallel and very twisted β sheet. β -hairpin turns connect the three pair of β -strands A' to A'', A''' to B and C to D. Residues 58–68 contain three consecutive reverse turns. These are stabilized by Pro 63 and two residues with positive ϕ conformations; Gly 67 and Gly 70. This region was well defined in both the MIRAS and solvent flattened maps. b, Schematic representation of the β -NGF subunit highlighting structurally important residues. Bold, absolutely conserved residues for all NGF and NGF-related sequences. Squares, buried residues in the β -NGF subunit with a relative side-chain solvent accessibility of less than 7%. The hexagons represent residues involved in the dimer interface and were identified from an examination of the β -NGF dimer using computer graphics. ~, Side-chain of the residue participates in a hydrogen

bond to either a side-chain or main-chain atom. The main-chain hydrogen bonds involved in the β -sheet structure are displayed as arrows, pointing in the direction of the donor to acceptor.

FIG. 3 *a*, Ribbon representation of the β -NGF dimer. The cyan and dark blue ribbons each represent a sub-unit. The S_γ atom for each half-cystine residue is also shown drawn as a sphere. *b*, Location of the variable sequences on the β -NGF structure; residues with nonconservative changes are indicated by their one-letter amino-acid codes. The residues are clustered in three β -hairpin loops (29–35, 43–48 and 92–98) and the reverse turn from residues 59–66. These are proposed to form important receptor-binding determinants that may contribute to the different high-affinity receptors found for NGF (left), BDNF (middle) and NT-3 (right). *c*, Electrostatic potential surface computed with the program DELPHI (ref. 38) and displayed with the program AAK at the level of the solvent accessible surface with a probe radius of 1.4 Å (courtesy of A. Nicholls and B. Honig, Columbia University). Blue represents positive potential, red negative and white neutral. The views show the β -NGF dimer (left) perpendicular to and (right) looking along the dimer twofold axis.



investigate receptor recognition determinants and through this the observed biological responses, such as neuronal survival. □

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The next cluster of differentiation (CD) workshop

Timothy A. Springer

One may participate in the next workshop by submitting monoclonal antibodies (mAbs) that react with molecules on the surface of human leukocytes, platelets or endothelial cells, by carrying out investigations on the mAb panels sent out by the workshop, or by attending the concluding meeting.

THE Workshop on Human Leukocyte Differentiation Antigens organizes international exchanges of mAbs, cooperative research on cell-surface molecules, and the CD nomenclature. Plans have just been laid for the fifth workshop, and I have been asked by the organizing committee to summarize these for members of the scientific community so they may understand how this important scientific undertaking works, how to participate, and how new features such as sections on adhesion structures, cytokine receptors, and endothelial cells broaden the scope of the workshop. This broadening of scope reflects an increasing emphasis in the workshops on the function of cell-surface molecules. There is no doubt that these workshops have substantially contributed to the extremely rapid advances that mAb technology has enabled not only on the serology but also on the molecular and cell biology of leukocyte cell-surface molecules. Extra dimensions about these molecules are revealed because scientists come together that are studying the same molecule under different guises — under different names, often on different cell types, and commonly from different points of view — as a surface antigen, a leukocyte subpopulation or malignant cell marker, a protein structure, a cDNA sequence, a receptor, an adhesion molecule, an enzyme, or a functional entity important in antigen-specific responses, host defence or homeostasis. The exchange of reagents — in the last workshop over 1,100 mAbs were analysed and over 500 laboratories participated — provides rich cross-fertilization for the field and encourages cooperation that extends beyond the workshop.

CD nomenclature

The CD nomenclature has been widely adopted, relieving the previous babel, yet few researchers know that CD stands for "cluster of differentiation", let alone the reference system and procedures that lie behind the nomenclature. Since the inception of the workshops, the primary criterion for grouping together mAbs that react with the same cell-surface structure has been reactivity with a panel of cell types. The percentage of cells positive for each antibody by indirect fluorescent

staining and flow cytometry is determined for purified leukocyte subpopulations and cell lines. A matrix of distances between antibodies, that is, the absolute difference in percentage reactivity for each pair of antibodies averaged over all cell targets, is used to construct a dendrogram revealing which antibodies have the most similar patterns of reactivity, or in other words form "clusters of differentiation". Reference mAbs are thus chosen that define each CD. Molecular characterization of the target antigen has revealed excellent agreement with this clustering method, and is now required for a final CD assignment. The CD designation originally referred to mAbs. This was considered an operational expedient, because in some cases CDs were assigned before molecular structures were known, and some structures are complex, involving carbohydrate determinants or alternative mRNA splicing. However, the CD designation has now become widely used to refer to the structures that are defined by CD mAbs.

How the workshop works

The workshop is divided into sections*, to increase efficiency and spread out the burden among the organizers of handling and distributing the large number of mAbs that are studied. The sections will be forums where antibodies will be gathered that are of interest to people working on a specific cell type or specific class of surface molecules. The antibodies do not have to be specific for a given cell lineage, but must react with a given cell lineage in the case of sections that focus on a specific cell type. Each section will sponsor a main panel containing unclustered mAbs and a few clustered reference mAbs. Where interest warrants, additional "CD panels" with collections of mAbs putatively reacting with the same, previously clustered CD molecule will be prepared. Some of the CD panels will be organized as "satellite workshops" by outside volunteers; those interested in a specific CD should apply to the workshop organizing committee and obtain its endorsement.

The first step in the workshop (January 1992) will be a call for antibodies. Researchers are invited to submit informa-

tion on antibodies they think would be interesting to study, particularly those that recognize previously unclustered antigens. Any antibody that reacts with leukocytes, platelets or endothelial cells, or activated or tumour cells of these lineages, is eligible. Careful selection of submitted antibodies is important to maximize the efficiency of the workshop, that is, the ratio of new CD assignments to antibodies analysed. This means that only well characterized mAbs can be accepted. Characterization should be both as to cell distribution and the structure or function of the target antigen; if only cell distribution is known, it should be carefully compared to that found with previously clustered mAbs using the Leukocyte Typing Database (see below). The organizers will also solicit submissions of specific mAbs; a preliminary tally identified over 50 cell-surface structures that have been cloned or identified with mAbs that as yet do not have CD assignments. At least two mAbs, from different laboratories, are required for a CD assignment, so information on related mAbs is encouraged when possible.

The next step in the workshop will be distribution of the mAb panels. Each section will aliquot the submitted mAb into 100–200 replicates of each panel, each containing roughly 100–200 mAbs. Panels will be sent to laboratories that volunteer to help with clustering, or carry out other types of scientific or clinical investigations. Those wishing to receive panels must submit a protocol describing the planned experiments. Priority will be assigned based on the contribution to the clustering and scientific goals of the workshop. Clustering studies may utilize (1) the traditional technique of immunofluorescent flow cytometry (2) immunostaining of tissue sections, a powerful method provided that standardized methods for reporting the staining can be incorporated for cross comparison between laboratories (3) immunoprecipitation (4) reactivity with products of transfected genes and (5) novel methods to be proposed. Basic science studies and clinical investigations using the antibodies can take many forms that are limited only by the imagination. To ensure that both submitters and testers of mAbs benefit in this collegial workshop

enterprise, there is an obligation to report results at the meeting that concludes the workshop, before they are communicated elsewhere. Selected contributions will be chosen for chapters in the workshop book.

Data that are gathered by researchers will be communicated back to the sections distributing mAbs and to a special section for data analysis. This will be analysed so that the new cluster assignments can be presented at the final meeting. This meeting (November 1993) will include presentations of studies carried out in each workshop section and a plenary scientific symposium.

Each workshop is summarized in a book, and for the third and fourth workshops, a computer database and program. The books contain a wealth of information on surface molecules and the mAbs to them¹⁻⁴. All the antibodies studied are listed and indexed, along with their contributors and addresses. This greatly facilitates obtaining mAbs from one's colleagues. The databases contain reactivities of each mAb with each cell type tested, valuable information not found anywhere else^{5,6}. The companion programs have many uses. For example, one may find strongly positive cell lines that would be well suited for purification of a particular antigen. Or, the cellular reactivities for one's own newly generated mAb may be added to the database, and the closest matches found with mAbs in the database. A database listing all CD molecules and their defining characteristics could be maintained for access through computer networks, much as is done for nucleic acid and protein sequences.

For further information on how to participate in the workshop, please write to Stuart F. Schlossman, Dana-Farber Cancer Institute, 44 Binney Street, Boston, Massachusetts 02115, USA. □

*Chairman, Stuart F. Schlossman; activation antigens, Roland Schwarting; adhesion structures, Timothy A. Springer; B cells, Thomas F. Tedder; cytokine receptors, Tadimitsu Kishimoto; data management, Stephen Shaw and Wally Gilks; endothelial cells, Michael A. Gimbrone; myeloid cells, Robert F. Todd; NK antigens, Jerome Ritz; platelets, Roy Silverstein; T cells, Laurence Bousmell.

Timothy A. Springer is at The Center for Blood Research, Harvard Medical School, 800 Huntington Avenue, Boston, Massachusetts 02115, USA. For more information, fill in reader service number 100.

Cellular research briefs

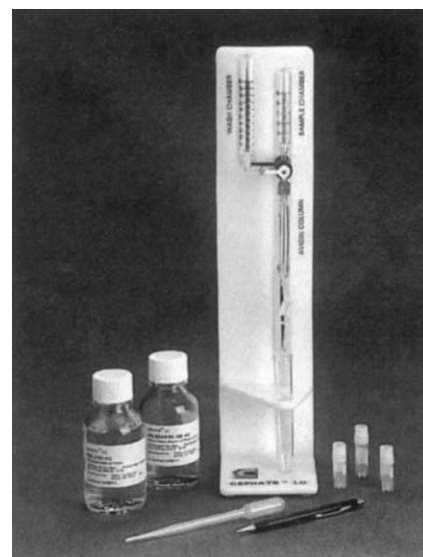
Over 350 companies will be showing off their wares at next week's 31st Annual Meeting of the American Society for Cell Biology to be held in Boston, Massachusetts, USA. Featured exhibits will include a new closed-loop mass cell culture device and an ultrasonic cell disrupter.

USING an indirect CO₂ conductance method, the specially adapted cell in the Malthus 2000 analyser can **detect and enumerate yeasts and moulds**, says Malthus Instruments (*Reader Service No. 101*). The cell measures conductance changes in a base solution brought about by absorption of CO₂ evolved by the growth of yeasts and moulds in the sample under test. Testing times can be cut down from seven days by traditional testing techniques to less than 24 hours when using the Malthus method, says the company.

Cell separators

CellMicroSieves from BioDesign are a **filter matrix of woven nylon filaments** specially formulated to be inert for biological research (*Reader Service No. 102*). Available in 11 different pore sizes ranging from 5 µm to 200 µm, CellMicroSieves are designed to provide a useful laboratory tool for the rapid, yet gentle, isolation of cells, organelles, or cellular debris. These specific-sized porous screens can be used to separate cells based on size, collect either cells or media without centrifugation, isolate single cells from dissociated tissue, or rapidly pass media in a bioreactor. As CellMicroSieves can be steam sterilized, they are reusable. Sheet, roll or filter-disk forms of CellMicroSieves are available.

To meet the increasing research interest in haematopoiesis and stem cells, and to facilitate the rapid selection of these rare cells from heterogeneous cell populations such as bone marrow, CellPro has developed the Ceprate LC (CD34) **laboratory cell separation system** (*Reader Service No. 103*). The Ceprate LC kit is a disposable cell separation system that utilizes avidin-biotin immunoaffinity to separate and enrich progenitor cells (CD34⁺) more quickly and easily than other currently available techniques, CellPro says. The system contains monoclonal antibodies, solutions, an avidin-gel column and other materials needed to purify those stem cells that are designated CD34⁺. Each kit can process up to 500 million cells in less than 3



CD34⁺ cell selection system.

hours, providing high purities and yields of CD34⁺ cells, CellPro says. The method is designed to provide a greater than 50-fold enrichment of colony forming cells that are able to differentiate into unipotent and multipotent cells (BFU-E, CFU-GM and CFU-MIX). The Ceprate LC system has also been used to select lymphoid subsets and appears to have broad applicability for selecting other cell populations. CellPro is using similar technology to develop a fetal cell concentrator. As part of a special introductory offer lasting until 31 December, CellPro is offering three Ceprate LC kits for \$499.50 (US).

Growth areas

With its space-saving design, the CellCube System developed by Costar represents a new concept in the **mass culturing of anchorage-dependent cells** (*Reader Service No. 104*). Each CellCube is an integral, self-contained culture module that is comprised of parallel tissue culture-treated polystyrene plates interspersed with media flow channels. Three sizes are available with growth areas of 21,250 cm², 42,500 cm² and 85,000 cm². To illustrate the compact nature of the system, a CellCube module equivalent to

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25 standard roller bottles measures just 9×9×3.5 inches, Costar says. Following seeding and growth phases, the CellCube will support monolayer densities exceeding 10⁷ cells per millilitre, depending upon cell type. To scale up production, four large size CellCubes can be run in parallel, providing 340,000 cm² of growth area. Support equipment includes a system controller and oxygenator, a circulation pump and a feed pump.

Millicell culture plate inserts with cellulose ester and transparent Biopore membranes are useful tools for the study of attachment-dependent cells and cell cultures in suspension. Now, Millipore offers Millicell inserts with a polycarbonate track-etched Isopore membrane — a very thin membrane with uniform straight pores (*Reader Service No. 105*). Isopore membranes allow rapid diffusion of macromolecules and their low-binding properties protect against the loss of precious proteins, Millipore says. Additionally, the membrane is designed to exhibit little or no background fluorescence when immunofluorescent or fluorescent stains are used.

Pulsing devices

The techniques of electrically-induced permeabilization and electrical cell fusion overcome some of the problems associated with the use of chemical agents or viruses. B. Braun offers an extensive line up of instruments for the **electrical transfection and fusion of cells**, namely the Biojet MI (electrical transfection) and the Biojet CF50 and Biojet CF100 (electrical cell fusion) (*Reader Service No. 106*). To complement this range of equipment and to provide added flexibility, the company offers a wide range of fusion chambers. The Biojet CF50 is designed to provide researchers with an economical introduction to the technique. The instrument is microprocessor-controlled and most of the process parameters are set digitally. After presetting the operational parameters, the fusion pulse can either be released manually or automatically. Additional design features of the CF50 include a "safe" load indicator and a special "protoplast fusion mode" that checks and limits the maximum voltage and can provide a precise adjustment of the voltage for larger cells.

The Virosonic 50 cell disrupter from VirTis is designed specifically to meet small laboratory sample processing requirements (*Reader Service No. 107*). Providing up to 50 W of high-intensity power, the Virosonic 50 can be used in a wide variety of ultrasonic applications, including the disruption of bacterial cells, viruses and spores, emulsification, dispersion of solids in liquid, acceleration of enzymatic and chemical reactions, and



Ultrasonic cell disrupter.

the degassing of liquids. The Virosonic 50, which is designed for use with an associated 0.125-inch diameter titanium microprobe, can be used to process sample volumes ranging from 0.2–40 ml in both wide and narrow vessels. A manual pulsing switch for intermittent operation is a handy addition, particularly where the build up of heat in a sample through continuous and prolonged use of the device would be a concern.

Enzyme immunoassay kits

According to Endogen, the company's second generation tumour necrosis factor (TNF) alpha ELISA can be used to **measure levels of human TNFα** — both natural and recombinant — in tissue culture supernatants, serum and plasma (*Reader Service No. 108*). The dual-antibody immunometric sandwich ELISA is designed for researchers studying the immunological effects of TNFα in cancer, AIDS, transplant rejection, rheumatoid arthritis and other immune disorders. The assay, which produces results in less than five hours, can detect as little as 5 pg of biologically active human TNFα per millilitre of sample, Endogen says. The assay is specific and does not cross-react with human IL-1 (α or β), IL-2, IL-3, IL-6, IL-7, IL-8, GM-CSF, IFNγ and recombinant murine TNFα. Users can run up to 43 samples in duplicate at one time or two partial plates on separate occasions.

Four new **eicosanoid microtitre assays** for the measurement of prostaglandin E₂, thromboxane B₂, 6-keto-prostaglandin F_{1α} and leukotriene B₄ can now be obtained from Advanced Magnetics (*Reader Service No. 109*). Handy pre-coated well strips provide both flexibility and consistency, and allow results to be obtained within 24 hours, the company says. The EIAs contain prediluted standards for ease of use and sufficient reagents for making 96 determinations, including standard curves.

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suppressor proteins, cell-cycle associated proteins, mouse cytokines and lymphocyte cell-surface antigenic determinants (Reader Service No. 110). The company is marketing a monoclonal antibody (mAb) product line against the retinoblastoma protein (human, rat, mouse and chicken) and p53 (wild-type, denatured and mutant forms). Applications include immunoprecipitation, immunoblotting, immunohistochemical staining (frozen and paraffin tissue sections) and flow cytometric analysis. PharMingen's product line to cell-cycle associated elements includes mAbs specific for p34^{cdc2} kinase (PSTAIR region and carboxy terminus), GAP protein, MAP2 kinase, EGF re-

ceptor and the S-phase cyclins. In addition, PharMingen offers a pair of mAbs that are optimized for IL-10 ELISAs. The individual antibodies (JES5-2A5 and SXC-1) are also effective at neutralizing IL-10 bioactivity. ELISA reagent sets (that is, capture mAb, detecting mAb and recombinant protein) are available for mouse IL-3, IL-4, IL-5, IL-6, IL-10, GM-CSF and TNF. Completing the line up is a comprehensive panel of antibodies against mouse lymphocyte cell-surface antigens. Plans are afoot to introduce mAb panels to rat and human lymphocyte antigenic determinants.

Hepatocyte growth factor (HGF), a potent mitogen for primary hepatocytes, is now available from Collaborative Biomedical Products (Reader Service No. 111). HGF has been localized in a variety of tissues and also has mitogenic effects on other cell types, including primary melanocytes and human mammary and monkey bronchial epithelial cell lines. Purified from human placenta, HGF is lyophilized from dH₂O, trifluoroacetic acid and bovine serum albumin. The company tests its HGF for biological activity in an assay measuring its ability to produce a 3–4-fold stimulation of DNA synthesis in HEPG2 cells over unstimulated controls. HGF is supplied as a membrane-filtered (0.2 µm) preparation, which the company claims is free from bacteria, fungi and mycoplasma.

C-C Biotech is distributing a range of **bovine serum albumins (BSAs)** manufactured by the Austrian biotechnology company, Haemosan (Reader Service No. 112). Different purity levels are offered including an acetylated, nuclease- and protease-free albumin for molecular biology research. This albumin is 99 per cent pure and contains no detectable levels of DNase, RNase, protease and immunoglobulins, says the company. A more recently developed albumin, slow virus inactivated, is designed to eliminate the potential threat of bovine spongiform encephalopathy and other viral contamination. The BSAs offered have been isolated by a new technique that is not based on conventional Cohn fractionation, and so no solvents are used. Multiple forms of BSAs are also offered to suit the needs of researchers. A new form, which results in a finished product that can be likened to "popcorn", has been developed using a proprietary freeze-drying technique. In this form, the BSAs are easy to handle and quick to dissolve, says the company.

Catalogues/custom services

Two new **publications** relevant to cell biology are newly available from Boehringer — a *Biochemicals for Cell Biology* catalogue and a manual entitled *Enzymes*

for Tissue Dissociation (Reader Service No. 113). Product groups covered in the catalogue include attachment, transport and growth factors, cytokines, serum-free medium supplements, hybridoma reagents, enzymes for tissue dissociation, and reagents for cell proliferation and signal transduction. Accompanying the



Boehringer's buyer's guide.

information on new product lines are details of important applications, physical properties, scientific background and supporting literature references. Boehringer's second product, the manual, provides details of the physical and functional characteristics of the many enzymes used for tissue dissociation, in addition to information on working procedures for selected cell types and an extensive reference list on literature describing the isolation of various types of cells.

Researchers studying receptor binding, cell binding, immunoprecipitation or any microquantification may be interested to hear that Multiple Peptide Systems is offering a new service, the **tritium labelling of custom-made peptides** (Reader Service No. 114). The company will custom synthesize a peptide sequence and then label to high specific activity (>50 Ci mmol⁻¹) with tritium covalently attached to the molecule. MPS says that the final compound will contain all natural underivatized amino acids and no foreign residues, such as iodinated amino acids. The average peptide will contain 2–3 tritium atoms per molecule and each is supplied with all the necessary quality-control data in a certificate of analysis. For convenience of handling and storage, labelled peptides are shipped in ethanol in special V-vials. □

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